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Aspirin in Type 2 Diabetes:

A survey of prescribing habits and Investigation of effects on Inflammation, Oxidative stress, Insulin Resistance and Endothelial Function.

Rajeev P Raghavan, MBBS MRCP (UK),

Collaboration with:

Academic Unit of Diabetes and Endocrinology,
Queen Alexandra Hospital, Portsmouth

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DECLARATION

Whilst registered as a candidate for the above degree, I have not been registered for any other award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

Signature:

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GLOSSARY

ACE= angiotensin converting enzyme

AEAC=Ascorbate equivalent anti-oxidant concentration (μM)

EUDRACT=European Union Drug Regulating Authorities Clinical Trials

FRAP= ferric reducing ability of plasma assay (assessing total anti-oxidant capacity)

HOMA= homeostasis model assay

HsCRP= high sensitivity C-reactive protein

HDL-C= high density lipoprotein fraction of cholesterol

LDL-C = low density lipoprotein fraction of cholesterol

NO= nitric oxide

PG= prostaglandin

PGI_2= prostaglandin I_2 or prostacyclin

POPADAD= prevention of progression of arterial disease and diabetes trial

PPI= Proton pump inhibitor (gastric acid suppressing medication e.g. Lansoprazole)

RAS= renin-angiotensin system

sVCAM-1= soluble vascular endothelial cell adhesion molecule type-1

TAOS= total antioxidant status assay (ABTS+ method)

SUMMARY OF STUDY RESULTS

a) Summary of observational study results

Aims: Aspirin is recommended in secondary prevention (SP) in diabetes and macrovascular disease. Recommendation in primary prevention (PP) remains controversial as does the dose of aspirin prescribed. To ascertain whether these controversies are reflected in clinical practice, we conducted a survey of healthcare professionals' views on aspirin prescribing in diabetes

Methods: An anonymous online survey consisting of 26 questions (Likert scale) covering demographic characteristics and aspirin prescribing habits in primary prevention and secondary prevention was circulated via email.

Results: 152 people responded with variable response rates: Primary care (96/152, 63%) - mixture of doctors/Diabetes Specialist Nurses; Secondary care were predominantly diabetes specialists (56/152, 37%).

Primary prevention (PP): 39/103(37%) did not routinely prescribe aspirin whilst 16/121(13.2%) would consider using aspirin in all diabetes patients as primary prevention. Using Chi-square contingency tables showed that there were differences when prescribing aspirin with regards to hypertension as a risk factor in primary prevention between primary care (20/68[29.4%] opting for aspirin) and secondary care (24/49 [48.98%], p-value-0.03) and doctors and nurses (38/60 vs 16/58, p=0.0009) and also with microalbuminuria - primary care vs secondary care (15/67vs 21/48, p=0.015), and doctors versus nurses (26/60 vs 11/59, p=0.004). Nurses were less likely to

prescribe aspirin as primary prevention in smokers (11/57[19.2%] vs 22/60 [36%]; OR-0.41 [0.16-1.03], p=0.042)

Secondary prevention (SP): Despite no contraindications 8/125(6.4%) would not give aspirin. 75mg/day or 300mg/day preferred doses in various settings. There were no statistically significant differences between primary and secondary care (62/73 vs 47/52 or 84.9% vs 90.4%, p=0.36) but doctors prescribed aspirin more often compared to nurses (59/67 vs 51/85 or 60% vs 88.1%, p=0.006).In patients with history of peptic ulceration respondents recommended a) use of PPI cover in PP-37/103(35.9%) and SP-60/103(58.3%), b) enteric coated aspirin PP-13/103(12.6%) and SP-11/103(10.7%), c) not use any anti-platelet agents in PP-53/103(51.5%) and SP-8/103(7.8%).

Enteric coated aspirin recommended by respondents as follows: Always-8/109(7.3%), sometimes-16.5%, occasionally-37.6%, and never-35.8%. 89/103(86.5%) had stated their patients had raised issues with them regards aspirin use. 27/103(26.2%) would definitely take aspirin themselves if they had diabetes. The differences were not significant either in a primary prevention setting or a secondary prevention setting when primary care was compared to secondary care but doctors were more likely to prescribe aspirin with PPI cover or in the enteric coated form compared to nurses (48/57[84.2%] vs 23/46 [50%]; OR=0.188 (0.067-0.511), p<0.001).

Conclusions: This survey confirmed that the controversy with regards to aspirin use particularly in primary prevention was reflected in a heterogeneous prescribing of aspirin in patients with diabetes. Further clarification and guidance on the optimum dose of aspirin in diabetes is required.

b) Summary of Interventional Results

Aims: To study the effects of aspirin at different doses on markers of oxidative stress, insulin resistance, dysglycaemia, endothelial function, and vascular inflammation in the primary prevention setting in a population with type 2 diabetes and high risk of cardiovascular disease.

Methodology: Following baseline assessment subjects had aspirin (75mg, 300mg, 3.6 gm) or placebo (with minimum 2 week washout) and pre-intervention and post-intervention assessment of markers for metabolic indices (Blood pressure, weight, BMI, Fructosamine, Lipids, Creatinine),oxidative stress (TAOS, FRAP, & Glutathione assays), insulin resistance (HOMA), vascular inflammation (HsCRP, sVCAM-1), and endothelial function (photoplethysmography).

Results: (reported in Mean±1SD or Median and Interquartile ranges) (See Table 28, P114)

17 Caucasians, 12 males, 5 females with age range between 40 and 75 years, completed the study. Mean age of the cohort was 57.4±9.1yrs (mean±1SD), with baseline characteristics summarized in Table-6 & Appendix B. Briefly HbA1c was 7.9±1.2%, blood pressure systolic-130.8±11.5 mmHg & diastolic-73.95±6.97 mmHg, total cholesterol-4.57±1.01 mmol/l, and HDL-C-1.13±0.46 mmol/l. At baseline TAOS concentration was 59.3±9.7 (ascorbate equivalent antioxidant concentration micromolar or AEAC (μM)), total glutathione-302.2±183.3μM, FRAP-0.86±0.23 (mM Fe II), HOMA-IR-1.41±1.04 Units, HOMA-S–76.27±45.20 %, Fructosamine-282.9±50.6 μM/l, RI-GTN- 7.17% (3.17-12.83), RI-Salbutamol- 18.5% (13-21.5), Hs-CRP (15

subjects)=0.66 mg/L (0.41 to 2.06 mg/L, Median & IQR), and sVCAM-1 (15 subjects)=487.04 ng/ml (IQR = 450.4 to 572.3). There was a trend towards significance for the TAOS assay with an increase in antioxidant capacity but it did not reach significance. Reduced glutathione (GSH): p=0.12, oxidised glutathione (GSSG): p=0.38, or Glutathione ratio (GSH:GSSG): p=0.367 were not significantly different following any of the interventions. Differences in FRAP were non-significant following any of the interventions. Measurements of insulin resistance (HOMA-IR), and insulin sensitivity (HOMA-S) seemed to improve with aspirin 75mgs/day & 300mgs/day but did not reach significance (see figure 18, 19). Neither the different doses of aspirin nor placebo had a significant impact upon glycaemic control (Fructosamine, P=0.39), endothelial function (photoplethysmography, RI-GTN-p=0.36, RI-Salb-p=0.46), Vascular inflammation (Hs-CRP-p>0.05, sVCAM-1>0.05), fasting glucose, BMI, blood pressure, or lipid parameters. Multiple regression analyses showed a good correspondence between the metabolic factors at baseline but were not repeated with different doses as there were no significant differences demonstrated with any of the parameters.

Conclusions: Aspirin at the doses studied and over the 2 week duration caused no significant changes in any of the markers studied. Good metabolic control (blood pressure, glycaemia, lipids), and widespread use of statins may be responsible for the lack of effect demonstrated.

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Introduction

Background:

Diabetes Mellitus encompasses a group of conditions with chronic hyperglycaemia associated with carbohydrate, protein, and fat metabolism and either a deficit in insulin secretion or action or both.(1) Until the discovery of insulin in 1922 by Banting and Best in Canada, this was essentially an untreatable illness with absolute or relative lack of insulin. This group of conditions has been recognised since ancient times (as far back as 1522 BC) in various cultures and civilisations. The association of weight loss from dehydration, polyuria, and polydipsia and the presence of sugar in the urine was first recognised and described by the Greek physician Arateus who coined the term Diabetes to describe "the melting down of flesh and limbs into urine". Until the 19th Century when tests were devised to identify the high sugar content in blood and then urine, diabetes was diagnosed using "water tasters" who picked up the high sugar content in the urine.

Paracelsus first recognised the serious and general implications of the disease in the 16th century. Hyperglycaemia is associated with complications in both the micro and macrovasculature causing long-term morbidity and premature mortality. It was subsequent work by Claude Bernard who worked on understanding the role of the pancreas and liver in digestion and glycogen metabolism, and in 1869 by Paul Langerhans who isolated the insulin secreting part of the pancreas called the Islets of Langerhans that helped further understanding of the importance of the pancreas in diabetes. The French physician Bouchardet recognised the effects of diet on improvements in some people with diabetes

when there were food shortages during the Franco-Prussian war in 1870, and advocated personalised dietary regimens.

The current diagnostic definitions for diabetes have been drawn up by the World Health Organisation (WHO) (1) and adopted in 2000, definitions which were adapted from proposals by the American Diabetes Association (ADA), and include either a fasting plasma glucoses (FPG) ≥ 7.0/mmol/l (whole blood venous glucose ≥ 6.1 mmol/l) or a random plasma glucose (RPG) ≥ 11.1 mmol/l (whole blood venous glucose ≥ 10.0 mmol/l) with symptoms. For epidemiological purposes either a fasting plasma glucose ≥ 7.0 mmol/l or a 2-hour plasma glucose ≥ 11.1mmol/l post formal oral glucose challenge (consisting of 75g carbohydrate load, e.g. Polycal) is considered diagnostic. On an individual/ clinical level however it is recommended that in the absence of unequivocal symptoms of hyperglycaemia or metabolic insult, the fasting or random tests are repeated on another occasion for confirmation (1). The World Health Organisation (WHO) criteria as outlined above have helped standardise the diagnosis of diabetes and made it easier for recognition of this disease entity.

The National Diabetes Data Group (NDDG) produced a consensus document in 1979 which standardized the nomenclature and definitions for diabetes mellitus and recognised 2 major classifications based on clinical description as "insulin-dependent diabetes mellitus" (IDDM) and "non-insulin-dependent diabetes mellitus" (NIDDM).

This document was endorsed by the WHO a year later. In June 1997, an international expert committee constituting members from the American Diabetes Association (ADA), and WHO released a report with new recommendations for the classification and diagnosis of diabetes

mellitus. The use of classification systems and standardized diagnostic criteria facilitates a common language among patients, physicians, other health care professionals and scientists.

The 4 most common types of diabetes in England and Wales are as follows-

- Type 1 diabetes
- Type 2 diabetes
- Secondary diabetes (from pancreatic damage, hepatic cirrhosis, endocrine
    - disease or therapy, or anti-viral/anti-psychotic therapy)
- Gestational diabetes (diabetes related to pregnancy).

Type 1 diabetes (T1DM) is an absolute insulin deficiency due to immune-mediated destruction of pancreatic β-cells. Typically this is associated with younger age of onset, leaner body mass, and the presence of circulating auto antibodies directed against the islet cells of the pancreas and with human-leucocyte antigen (HLA) related genetic markers.(2)

The insulin deficiency of T2DM is progressive, such that hyperglycaemia usually worsens inexorably over a variable time scale (usually years but could be months or rarely days), leading to progressive treatment failure and requiring continued escalation of blood glucose lowering therapy. The worsening insulin deficiency with age also means that diabetes can appear in elderly people who are thin and as a consequence in some middle aged people, the condition can be difficult to distinguish from slow onset Type 1 diabetes or latent autoimmune diabetes in adults (LADA). In people whose hyperglycaemia has yet to be treated, glucose metabolism may be sufficiently disturbed to cause symptoms, typically of

polyuria, thirst, weight loss and fatigue. Diabetic ketoacidosis the hallmarks of which are hyperglycaemia, ketosis, acidosis, dehydration, and in severe cases diabetic coma (as a direct result of severe insulin deficiency) (3) is uncommon in Type 2 diabetes unless exacerbating factors (infection, drugs) are present, but relative insulin deficiency and similar compounding factors such as infections, drugs, and noncompliance can lead to a related state called hyperosmolar hyperglycaemic state, shortened as HHS (previously known as hyperosmolar non-ketotic state or HONK) (4) The key features of HHS include very high glucose levels, mild or absent ketosis, increased osmolality of the plasma, and altered sensorium or neurological deficits.(4) In broad terms the principles of treatment include replacement of insulin deficit and fluid loss and addressing the precipitating cause. Despite modern advances and standardisation of management these conditions can still lead to high morbidity and mortality.

In view of the problems of maintaining euglycaemia associated with increasing insulin deficiency, the degree of hyperglycaemia occurring in some individuals over time can give rise to complications involving small vessels down to the capillary level, so called 'microvascular' complications of diabetes. Chief among these complications are retinopathy (ultimately could result in blindness), nephropathy (ultimately requires dialysis or renal transplantation), and neuropathy (resulting in lower limb amputation, neuropathic pain and erectile dysfunction). The lead time from onset of hyperglycaemia to the development of these complications is variable but usually takes at least 5 years or more to develop. Given that impaired glucose regulation (IGR or prediabetes) may precede development of type 2 diabetes by several years (1) and that the latter may have been latent for several years, at

diagnosis, microvascular complications may already be present. In contrast there is no latency for type 1 diabetes and therefore such complications are a function of time and degree of control. However the burden from microvascular disease is probably less in type 2 diabetes due to premature death caused by cardiovascular disease.

The links between Diabetes and Cardiovascular disease

Diabetes (both type-1 and type-2) are increasing worldwide and the latter in epidemic proportions in association with the alarming increase in obesity (5; 6) T2DM is set to affect 366 million people worldwide in the coming decades.(7) Type 2 diabetes has been long been noted for an increased incidence of cardiovascular disease.(8) The large vessel or "macrovascular" complications can manifest not just as coronary artery disease (myocardial infarction, angina), but also as peripheral artery disease (leg claudication, ulceration, & gangrene) and carotid artery disease (cerebrovascular disease, dementia). People with Type 2 diabetes have the same risk of a cardiovascular event as someone without diabetes who has already had their first cardiac event;(9) people with diabetes and a previous cardiovascular event are at an even greater risk (3-10 times) compared with the background population based upon several studies.(10-12) Accordingly management of cardiovascular risk factors plays a pivotal part in care of people with Type 2 diabetes and has an extensive evidence base.

The management of traditional vascular risk factors such as hypertension and dyslipidaemia has been successful in reducing the development or progression of cardiovascular (CV) disease (13) but has not ameliorated this complication. This situation of multiple vascular risk

factors and complications leads to several targets for reduction of risk and improvement of health in people with Type 2 diabetes. These targets for management include obesity, activity levels, plasma glucose control, blood pressure control, blood lipid control, reduction of thrombogenicity, laser therapy for retinopathy, drug therapy to delay advancing nephropathy, local foot care, and symptomatic treatments for various types of neuropathy. As a result diabetes care is typically complex and time consuming. One of the chief aims of diabetes care is early intervention aiming to minimise cardiovascular risk as this is key to reducing morbidity and mortality associated with diabetes.

Novel markers of vascular risk that have received attention include endothelial dysfunction (a determinant of vascular tone) ,(14) vascular inflammation ,(15) oxidative stress, (as reviewed here by Pitocco et al,(16)) and insulin resistance .(9) In particular researchers have focused upon the links between vascular tone and inflammation, oxidative stress, insulin sensitivity and the aetiology and pathogenesis of diabetes.(17)

The metabolic syndrome, atherosclerosis, and cardiovascular disease

Type-2 Diabetes (T2DM) which is the focus of this study is the more common type (representing over 90% of the total in the UK) and demonstrates a dual pathology with reduced insulin sensitivity/ increased insulin resistance and an initial variable phase of increased insulin production followed gradually by inadequate compensatory insulin secretion due to pancreatic beta cell failure.(18) The association of diabetes with cardiovascular disease is well established (13) but the underlying pathophysiology is still not fully understood. Insulin resistance, oxidative stress, and vascular inflammation appear to be

key factors. Insulin resistance is usually associated with excess body weight or obesity, and exacerbated by overeating and inactivity. It is associated with and thought to engender a clustering of risk factors that include raised blood pressure, a disturbance of blood lipid concentrations (typically low HDL cholesterol, high triglycerides, and a preponderance of small dense LDL particles), dysglycaemia, and central obesity or visceral adiposity (increased waist circumference). The clustering of these risk factors has previously been described by the umbrella term 'metabolic syndrome' and was first described by Gerald Reaven (19) (see Table-1).

Table -1: Definition of the Metabolic Syndrome according to the International Diabetes Federation, 2005 (from Zimmett P et al. 2005) (20)

Characteristic	**Definition**
Obesity ***Plus* any 2** of the following	Defined by waist-circumference-ethnicity specific (or if BMI > 30 when former need not be measured)
Raised Triglycerides	≥150 mgs/dl (1.7 mmol/l) **or** being specifically treated for this condition
Reduced HDL-cholesterol	<40 mg/dl in males (1.03 mmol/l) <50 mg/dl in males (1.29 mmol/l) **or** being specifically treated for this condition
Raised blood pressure	Systolic >130 mmhg Diastolic > 85 mmHg **or** treatment for previously diagnosed hypertension
Raised Fasting plasma glucose	Fasting plasma glucose > 100mg/dl (5.6 mmol/l) Or previously diagnosed type-2 diabetes If above 5.6 mmol/l (100mg/dl) then OGTT recommended but not strictly so for diagnosis of metabolic syndrome

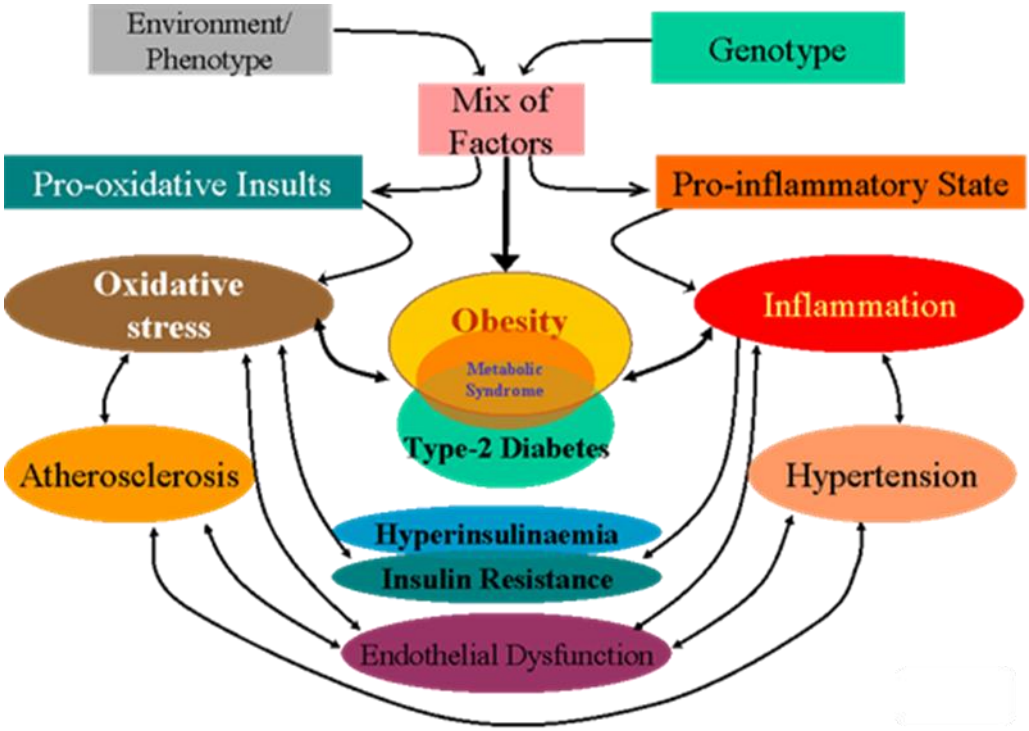
Extensive research has shown a definite risk of progression from metabolic syndrome to type-2 diabetes but the definition, scope, and validity of this condition has been called into question.(21) The main factors behind the scepticism has been due to varying definitions

proposed by sources such as the National Cholesterol Education Programme (NCEP III), International Diabetes Federation (IDF), and the WHO, concern regarding varying phenotypes, utility of the term in clinical practice, and also the lack of a unifying aetiopathology for the individual components of the syndrome. Moreover the benefits of looking for such clustering, rather than focusing on the individual risk factors in cardiovascular risk reduction has been questioned. However further work in this area has led to the term metabolic syndrome being accepted in medical practice and acknowledges the fact that the term meets the definition of the word "syndrome" as the clustering of the individual components occurs more often than would be attributable to chance and the basis for the clustering is undefined. In a study of 2051 subjects defined as having metabolic syndrome, application of three different sets of criteria over a ten year period demonstrated no significant differences in their risk for cardiovascular disease and diabetes (22) but some differences in sensitivity were evident between the criteria studied. A recent international consensus has been thrashed out to ensure global uniformity in definition criteria while allowing for variation in defining ethnicity specific criteria such as waist circumference.(23)

In 1995, Stern proposed the Common Soil hypothesis (24) linking atherosclerosis, inflammation, and the metabolic syndrome (20) and there has since been an abundance of evidence to support these interlinked conditions and the complex pathophysiology underlying the metabolic syndrome. This is summarised in Figure-1. It is recognized that endothelial dysfunction is a key feature of the metabolic syndrome, atherosclerosis and type-2 diabetes. There is also extensive work linking endothelial function to oxidative stress and insulin resistance.(14) demonstrated the importance of endothelial functional integrity in coronary

ischaemia and atherosclerosis, (14) the former depending upon metabolic vasodilation via tonic release of endothelial NO and vasodilator prostanoids.(25) The role of macrophages and circulating leukocytes in endothelial injury and atherosclerotic plaque development needs to be stressed.(26) Impaired NO production, increased peroxynitrite formation, a pro-inflammatory milieu, and endothelial damage are some of the mechanisms leading to impaired vasodilatory capacity and endothelial dysfunction.

Figure-1: Schematic Illustration of links between pathophysiological factors underlying metabolic syndrome and type 2 diabetes. (reproduced with permission from Raghavan.R et al, BJDVD, 2006)



Oxidized LDL causes a reduction in endothelial-cell NO concentration as demonstrated by Cominacini et al. (2001),(26) providing a link between oxidative stress and endothelial dysfunction. Oxidized LDL also induces endothelial adhesion molecule expression, an

important factor in monocyte adhesion.(27) Elevated plasma ICAM-1 levels correlate with increased risk of coronary events even in an apparently healthy population (28) as does elevation in CRP levels, with impaired vasoreactivity (29) being proposed as one mechanism for the latter. These underline some of the interlinked pathways illustrated.

Developments in the pathophysiology of type 2 diabetes and cardiovascular disease

Hypothesising about disease origin –

Although some of the mechanisms underlying the metabolic syndrome, type-2 diabetes and co-existing cardiovascular disease are well defined; the primary defect is not known. In the last couple of decades a substantial body of work has suggested that oxidative stress, insulin resistance, endothelial dysfunction (in the peripheral arterial bed) and more recently inflammation might be acting synergistically, to promote the development of the metabolic syndrome and subsequent pathology. (16; 17)

Oxidative stress impinges upon endothelial function (Lipinski, 2001)(16) and mitochondrial metabolism and is a major culprit in the complex web of factors causing and propagating the syndrome of type-2 diabetes.(17) Although its origin is still debated, it is a well-documented phenomenon in atherosclerosis and diabetes; (30) to the extent that oxidative stress and formation of advanced glycation end products (AGE) are said to drive many of the pathological processes in these conditions. There is evidence favouring the fact that the balance between pro-oxidant properties of peroxynitrite formed from NO and anti-oxidant

properties of plasma (31) and nitric oxide, (32) determines the progression of pathology in atherosclerosis. There is an increased tendency for formation of reactive oxygen species (ROS) in type-2 diabetes, secondary to glucose auto oxidation, non-enzymatic protein glycation, and the interaction of AGE with specific endothelial receptors. (33; 34) Rapid inactivation of endothelium-derived vasodilator nitric oxide (NO) is caused by vascular superoxide anion..(35) Nitric oxide combines with superoxide intravascularly to create peroxynitrite, a cytotoxic molecule, which then oxidizes LDL.(36) Oxidized LDL independently predicts progression of atherosclerosis (37) and in turn may affect endothelial vascular tone by increasing superoxide generation and causing reduction in NO levels.(25) These observations suggest that oxidative stress may be the "common soil" for these processes.(38)

Chronic sub-clinical inflammation is widely being recognised as a key component in the aetiology of macrovascular disease and possibly even the metabolic syndrome and type-2 diabetes [IRAS study].(39) A rise in inflammatory markers in conjunction with insulin resistance was demonstrated in the Insulin Resistance & Atherosclerosis (IRAS) study .(39) Similarly Ridker et al. (40) have shown a strong association between elevated C-Reactive Protein (CRP) and atherosclerosis and also elevation in CRP and interleukin-6 (IL-6) levels being predictive of development of diabetes.(41) CRP seems to be helpful for risk-stratification in all stages of the metabolic syndrome,(42) although there is some evidence to suggest that complement C3 maybe a better marker of subclinical disease (43) and that CRP is more likely a "process marker" for factors causing atherosclerosis.(44) However more recently, the JUPITER study (40) showed that lowering CRP can cause reduction in

cardiovascular disease and Clapp et al. (2004) (45) showed that inflammation can cause endothelial dysfunction, reduced vascular NO bioavailability and increase oxidative stress, which may alternatively suggest inflammation as the initiating factor

It has been shown that salicylates or their metabolites produced at sites of inflammation may have an anti-oxidant role and enhanced anti-inflammatory capacity.(45) However several long-term studies have failed to find an antioxidant with a significant impact upon morbidity and mortality.(46-48) This challenges the contention that oxidative stress is the key component in the pathophysiology of the metabolic syndrome, macrovascular disease, and diabetes. The findings of a pro-inflammatory state in obesity, ingestion of a mixed meal being pro-inflammatory,(49) and raised inflammatory markers being directly linked to obesity rather than insulin resistance or dysglycaemia,(50) all make a case for inflammation being a possible factor inducing or driving oxidative stress.

The role of Nuclear Factor -kappa Beta (NF- κB) in cardiovascular disease and diabetes

In-vitro and in-vivo research has implicated fatty acid-dependent activation of the serine kinase IKKβ, which plays a key role in tissue inflammation, in the pathogenesis of insulin resistance.(51) NF-κB is an inducible eukaryotic transcription factor of the *rel* family, which is critical in regulating transcription of specific genes, most of which are involved in the immune/inflammatory responses, infection, and induction of various agents including interleukin-1&6, and adhesion molecules. In-vitro and in-vivo studies have shown links

between NF-κB activation and an increase in levels of adhesion molecules like VCAM-1, ELAM-1,(52) neutrophil transmigration,(53) and platelet-endothelial interaction/adhesion.(54) All of these are factors which play an important role in vascular inflammation and atherosclerosis.Table-2 outlines studies linking NF-κB to various processes in the pathophysiology of metabolic syndrome and type-2 diabetes. In addition, it has been shown that NF-κB controls the cytokine network and inhibition of NF-κB causes a decrease in cytokine activation. Yuan and colleagues (55) (2001) have also demonstrated that using salicylates decreased diet induced insulin resistance in the liver and skeletal muscle tissue of rats via inhibition of the NF-κB pathway. Mixed meal ingestion has been shown to cause an increase in NF-κB activation leading to a proinflammatory state.(49)

Table-2 :Nuclear factor – kappa beta, aspirin and diabetes		
Study	**Mode**	**salient points**
Kopp & Ghosh 1994 (56) Yin.M et al. 1998 (57)	in-vitro in-vitro & in-vivo (animal model)	aspirin& salicylate inhibit Nf-κB activation
Chen.C. et al. 1995(53)	in-vitro	Ikk-B inhibition → *decreased* VCAM-1, ICAM-1, & E-selectin
Pierce.J et al. 1996(52)	In-vitro (human cells)	aspirin *inhibits* Ikk-B activation & neutrophil transmigration
Kim.J et al. 2001 (54)	in-vivo (rats)	Fat-induced Insulin resistance decreased by salicylates via Nf-κB pathway
Yuan.M et al. 2001(55)	in-vivo (mice)	Ikk-B disruption by salicylates → *decrease* in diet-induced insulin.resistance
Gawaz M et al. 2002 (58)	In-vitro(human cells)	Platelet-endothelial interaction→ *increase* in MCP-1 & adhesion via NF-κB
Ripudaman.H et al. 2002 (51)	in-vivo (human)	high dose aspirin *improves* insulin sensitivity via NF-κB pathway
Matsunaga.T et al. 2003 (59)	in-vitro	endothelial cells exposed to Oxidized-LDL → Nf-κB activation

Adapted with Permission from Raghavan R et al., 2006 (60)

Pharmacology pointing to pathophysiology

The above findings suggest that decreasing both NF-kappa B activation (in liver, muscle, and adipose tissue) and inflammation, could lead to amelioration or resolution of the metabolic syndrome and possibly the diabetic state. The significance of this is underlined by the anti-inflammatory and anti-oxidant properties of glitazones (49) and insulin, with reduction in

levels of inflammatory markers and in ROS generation; effects thought to be mediated by NF-Kappa B inhibition through increasing levels of the inhibitor IKK-B (61) (see also Table 3). Aspirin in high dose (51) in a small group of subjects resulted in a reduction in insulin resistance and dysglycaemia evidently via the same pathway.

Table-3: Effect of various pharmacological agents/ lifestyle factors on pathophysiology of diabetes and dysmetabolic cardiovascular disease in clinical studies. (ACE=angiotensin converting enzyme, ARB=angiotensin receptor blocker, WOSCOPS=west of Scotland coronary prevention study [65], CAPP=Captopril prevention project [66], HOPE=Heart outcomes prevention evaluation study [67]).

Interventions	Oxidative Stress	Insulin Sensitivity	Endothelial dysfunction	Inflammation & adiposity	Progression to T2DM
Insulin	↓	---	↓	↓ CRP, NF-kB, Cytokines, PAI-1	?
Metformin	?	↑	↓ ?	↓ MCP-1	Retarded
Glitazones	↓	↑	↓	↓ CRP, NF-kB, Cytokines	Retarded
Statin	↓	---	↓	↓ CRP, NF-kB, Cytokines	Retarded (30% WOSCOPS)
ACE-Inhibitors	↓	↑	↓	↓ CRP, NF-kB, Cytokines, PAI-1	? (11-30% CAPP, HOPE)
ARBs	↓	↑ ?	↓ ?	↓ CRP, NF-kB, Cytokines, PAI-1	?
Aspirin	↓	↑ ?	↓	↓ CRP, NF-kB, Cytokines	?
Wt Loss/Exercise	↓	↑	↓	↓ CRP, NF-kB, Cytokines	Retarded
Obesity	↓	↑	↓	↓ CRP, NF-kB, Cytokines, PAI-1	↑

Reproduced with permission from Raghavan R et al., British Journal of Diabetes and Vascular Disease, 2006 (60)

NF-κB seems to influence various key inflammatory and immune pathways and has been linked to cancer and Adult Respiratory Distress Syndrome.(62) It acts as a transcription factor for various proteins including VCAM-1 and ELAM-1, which are responsible for monocyte adhesion to endothelium (63) and inhibiting NF-κB activation reduces monocyte adhesion.(64) Similarly many other drugs used in the treatment of atherosclerosis,

hypertension, and the metabolic syndrome exert some influence on the NF-κB pathway (see Table-3).

Salicylates and Aspirin

Salicylates were first isolated more than a hundred years ago from the bark of the willow tree, which has been used for its anti-inflammatory properties from ancient times. After being acetylated in 1879 and made less acidic, the resultant product acetylsalicylate (acetylsalicylic acid or ASA) was marketed successfully for Bayer as aspirin. Aspirin has been the subject of research and debate throughout its history. The worldwide consumption of aspirin is astounding and yet there are assertions that it may be underutilised in high-risk populations where it may be most needed. The mechanism(s) of action, function(s), and appropriate use of aspirin continues to be debated. Although aspirin has been unequivocally advocated for the prevention of ischaemic heart disease,(68, 69) its use in diabetes is not fully clarified.(70) Given the varied aetiological elements in the development of type-2 diabetes and ensuing cardiovascular risk, the role and dose of aspirin in this high-risk population may need to be redefined. This MD thesis examines the effects of aspirin upon processes associated with the development of cardiovascular disease in type 2 diabetes.

Exploring "established" facts on Aspirin use and Diabetes

The latest American Diabetes Association statement on the use of aspirin in diabetes (71) advocates the use of 81-152 mg of aspirin as primary prevention in any patient with diabetes over a certain age and with an additional risk factor (for example family history,

dyslipidaemia etc.). This replaces previous advice which has been confusing in clinical practice as to the target population and the point of initiation. The appropriate dose is also given some leeway with a range rather than a specific dose. This is however more focused compared to previous guidance from the same organisation which recommended a dose range from 81mg to 325mg, with 300mg dose for a longer duration advised only in the acute setting (for example post-infarction). The European task force has advocated the use of 75mgs/day as primary prevention for patients at high risk (diabetes, well-controlled hypertension, and males at significant risk of CVD).(72) Routine use was not recommended in all high-risk populations.(73) Diabetes UK in its guideline published in 2009 echoes this advice and recommends risk evaluation at the individual level before initiating therapy in primary prevention.(74). This takes into account further evidence (69, 75) suggesting that aspirin due to the increased risk of GI bleeding may not be beneficial in all patients with diabetes without a history of cardiovascular disease.

A study examining the use of aspirin before (cohort data from UKPDS) and after the ADA and Joint British Societies recommendations in the early part of the decade, showed an increase in the percentage of people without pre-existing CVD who were on aspirin (17 to 31%).(73)Similar findings have been noted in other international communities.(76-78) This represents sub-optimal prescribing when taking into account the advice on aspirin use in diabetes at the time. This finding perhaps partly reflects the uncertainty that exists in the minds of healthcare professionals regarding criteria for aspirin use in diabetes.

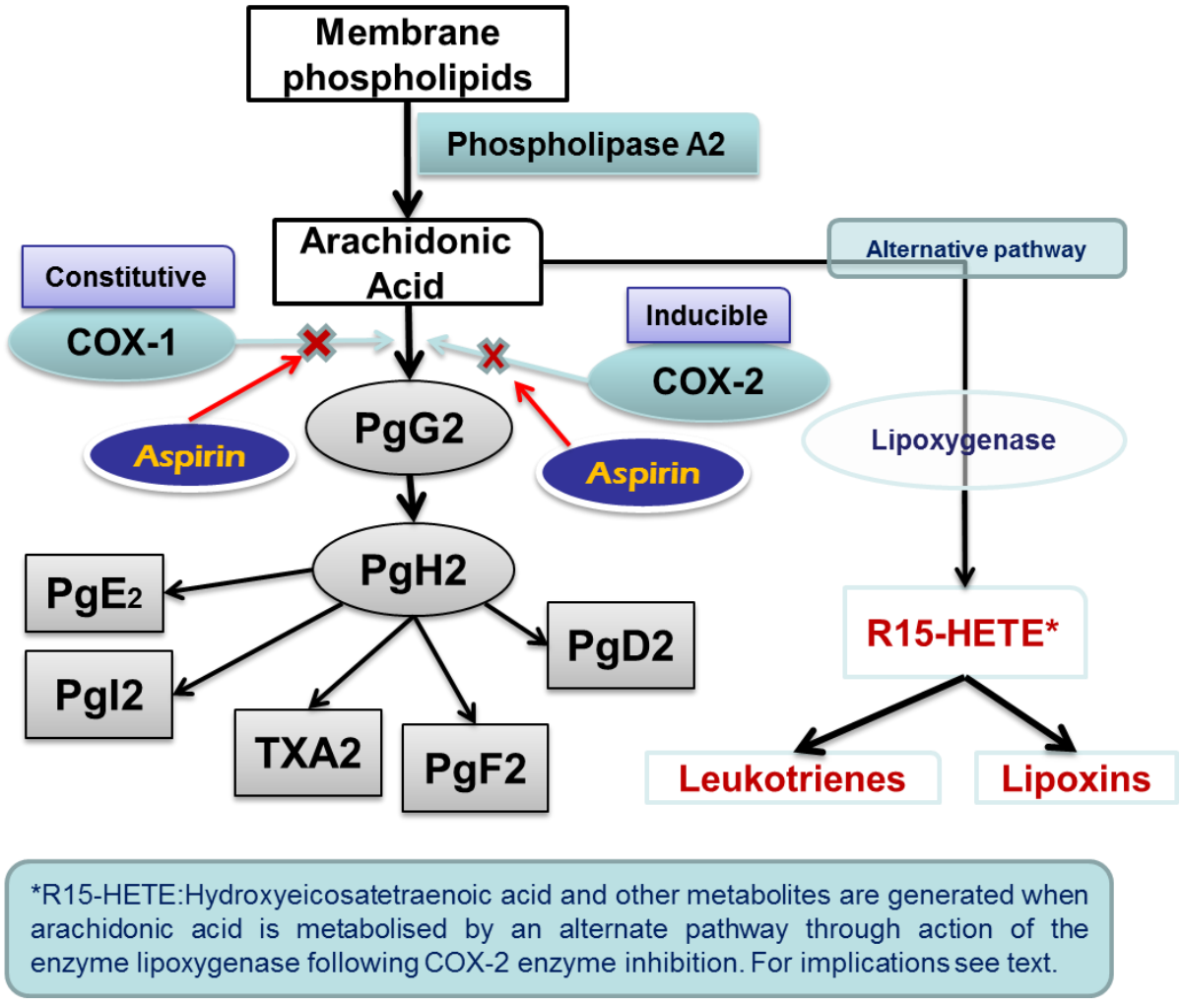
Advice may change in the future given the emerging concept of aspirin resistance (discussed below), the recognition of abnormal platelet activation and platelet response in diabetes, and other mechanisms of action attributed to aspirin at differing doses.

Aspirin in cardiovascular disease

The role of aspirin in the secondary prevention of cardiovascular disease is unequivocal. It is used globally for secondary prevention of cardiac, cerebrovascular, and other acute ischaemic events. Aspirin was found to reduce the likelihood of secondary events by an impressive 22% in all trials, with a 25±4% reduction following a prior MI and 46±7% following unstable angina, with lesser but significant effects in secondary prevention of stroke and other high risk vascular events.(68, 69) Data for Aspirin use in primary prevention especially in diabetes is scarce (just a 7% reduction in cardiovascular events demonstrated) despite several studies [PPP,(70) HOT,(79) etc.] and meta-analyses attempting to address this question.(68, 69) But given the confounding factors present in each study a case for "no benefit" has probably not been answered. In the ETDRS study (80) for example, 538(40%) patients in the treated group had a HbA1c greater than 10% (platelet glycation interferes with acetylation and antiplatelet effect (81)), a 5% per year attrition in adherence to study medication over 5 years, and a significant level of hypertension, which could explain the reduction in CV mortality not reaching significance. (See Table-4)

Inhibition of platelet cyclo-oxygenase (COX-dependent effect) leading to reduced platelet aggregability and plaque/thrombus formation remains the most widely accepted mechanism of action of aspirin in this setting (82) (see figure-2).

Figure-2: Model of prostanoid production, blockade of the COX-1 pathway, and subversion of COX-2 enzyme pathway by aspirin. (COX= cyclooxygenase enzymes, PG = prostaglandin, TXA = thromboxane). Adapted from Mitchell & Warner, Br.J.Pharmacol, 1999.(83)



*R15-HETE:Hydroxyeicosatetraenoic acid and other metabolites are generated when arachidonic acid is metabolised by an alternate pathway through action of the enzyme lipoxygenase following COX-2 enzyme inhibition. For implications see text.

Aspirin – Novel mechanisms and effects:

Novel actions of aspirin

Aspirin has long been in use as an anti-inflammatory, anti-pyretic, and analgesic in higher doses, although it is now well established that inhibition of platelet aggregation is achieved at quite small doses (with platelet cyclooxygenase inhibition nearly complete at even 37.5mg of aspirin (84). Traditionally salicylic acid and its congener aspirin have been seen as cyclo-oxygenase enzyme inhibitors (82) and by inhibiting platelet cycloxygenase reduce their tendency to aggregate and thus reduce the risk of thrombosis and plaque formation. However several studies with aspirin raise doubts that this is the sole mechanism by which these benefits are derived. Aspirin affects several pathways including those important to endothelial functional integrity such as the NF-κB pathway (see table-2). Various agents including NO, Angiotensin-2, and TNF-α act via up-regulation of this pathway in the endothelium (85) and by up-regulation of p53.(86) Aspirin inhibits both these pathways and reduces production of cytokines and adhesion molecules thus preventing leukocyte adhesion and transmigration.(52)

Aspirin reduced superoxide generation and improved vasorelaxation in normal and hypertensive rat models in a dose-dependent manner.(87) These effects seemingly retarded development of hypertension. Low dose (100mg enteric coated) aspirin seemed to exhibit a capacity to increase anti-oxidant capacity of plasma even on short-term administration despite no significant effect upon markers of anti-oxidant status and lipid peroxidation.(88) Conversely thromboxane biosynthesis which is resistant to aspirin can be caused by increased oxidant stress as demonstrated in unstable angina by Cippolone and colleagues. (89) Aspirin and its metabolite gentisic acid (90) interfere with LDL oxidation, (91) a process, linked to

severity of atherosclerosis (92) and to initiation of ROS generation via the superoxide pathway leading to endothelial injury. Potentially this is another mechanism behind aspirin reducing atherosclerosis.

The current emphasis upon vascular inflammation as a potent factor in the pathogenesis and progression of atherosclerosis, metabolic syndrome, and type-2 diabetes strengthens the premise that aspirin may be acting on non-platelet factors to influence outcomes.(93) Interestingly studies have shown that salicylates, which are weak suppressors of the COX isoenzymes, share the same profile of anti-inflammatory activity as aspirin thus challenging the contention that aspirin acts exclusively via the COX pathway [Multicenter salsalate/aspirin study group (94)]. An exciting development is the discovery of aspirins ability to acetylate the active site of cyclooxygenase enzyme in endothelial and epithelial cells "triggering" synthesis of lipoxins (also called aspirin triggered lipoxins or ATLs) and epi-lipoxins. (95) As outlined in figure-2 there is subversion of arachidonic acid metabolism to production of R15-HETE which is then converted by lipoxygenase enzyme to 15-epi-lipoxin-A4 or B4.(96) Production of such metabolites is a feature unique to aspirin and salicylates among the NSAIDs and these metabolites may exhibit an anti-oxidant role and enhanced anti-inflammatory action.(97) Both in rodents and humans administration of aspirin at 81mg/day was shown to increase plasma and urine levels of 15-epi-LXA4 and decrease thromboxane levels.(98)

Aspirin and sodium salicylate are both known inhibitors of NF-κB and this may be the mechanism by which they exert their anti-inflammatory effects. Moreover Cianferoni &

colleagues (99) showed that both the above agents inhibited Interleukin-4 secretion and gene expression (a cytokine involved in anti-inflammatory and immune responses) in human-T lymphocytes, independent of either prostaglandin synthesis or NF-κB inhibition.

Even as far back as 1877 Ebstein noted that acetyl salicylic acid (ASA) decreased glycosuria, a fact that has been largely ignored since. Interestingly, a recent study demonstrated that aspirin, in very high doses, has an insulin sensitizing effect, and that this action was chiefly mediated through the NF-κB pathway (51; 55). In support of this observation is work in-vivo demonstrating that aspirin prevents fat-induced insulin resistance in rats.(54) Increases in fibrinogen, impaired fibrinolysis and elevated PAI-1 levels are known pathophysiological factors in cardiovascular disease and are particularly important in the diabetic population. [NHANES III (100)][IRAS study (101)]. Aspirin has been shown to enhance fibrinolysis via acetylation of fibrinogen.(102)

Given the links between insulin resistance, glycaemic control, endothelial dysfunction and oxidant stress (for example in hypertension and/or diabetes), the beneficial effects of aspirin especially in high risk populations may be attributed to multiple actions and each of these mechanisms may be more evident at different concentrations of aspirin in-vivo.

Aspirin and endothelial function –

In addition to diabetes and atherosclerosis, the endothelium is involved in other disease processes such as hypertension. It has been noted that angiotensin-2 plays an important part in atherosclerosis and one of the mechanisms apart from increasing peripheral resistance seems to be by increasing expression of adhesion molecules and causing endothelial

activation, which is inhibited by aspirin.(85) The finding that aspirin treatment leads to production of a class of molecules called resolvins, which are anti-inflammatory (103) further underlines the vascular protective effects of aspirin. Interestingly in an in-vitro model aspirin increased NO production in platelets via cNOS (constitutive Nitric oxide synthase or NOS III); an effect not seen in endothelial cells, raising the possibility that this may have a role in preventing thrombosis and platelet aggregation.(104) However a recent study using human endothelial cells found that aspirin increased NO production.(105)

When endothelial cells exposed to a high-glucose environment were studied, aspirin caused an increase in endothelial NO production.(106) Grosser et al. (107) further demonstrated the cytoprotective action of aspirin via eNOS and cGMP pathway, all of which raises the possibility of aspirin exerting variable effects in different metabolic environments. Kharbandha et al. (108) showed that aspirin prevents endothelial dysfunction in an inflammatory milieu and it has also been shown to attenuate endothelial dysfunction in atherosclerosis.(109) Some possible mechanisms for this effect could include:

- The potent anti-inflammatory effects of aspirin triggered epi-lipoxin-A4 (LXA-4) (95) (see figure-2) and
- Decrease in neutrophil/leukocyte adhesion and interaction with endothelium by aspirin.(110)

Aspirin inhibits monocyte adhesion to endothelium, an additional action not exhibited by traditional non-steroidal anti-inflammatory agents (NSAID-for example ibuprofen) and this effect could result from ingestion of a single oral dose of 500mgs.(27) In a trial involving

patients with chronic stable angina, Ikonimidis et al. (111) showed that aspirin can reduce levels of interleukins and MCSF, each of which has a role in the progression of atherosclerosis and endothelial injury.(112, 113) Aspirin has caused reduction in CRP and IL-6 levels in one study (41) while Feldman et al. (114) found no effect, in healthy individuals.

In addition aspirin at a concentration of 30μM was found to allosterically inhibit endothelin-A receptors thus antagonising endothelin-1, a potent vasoconstrictor.(115, 116) Endothelin is involved in various pathological processes including coronary spasm, insufficiency and infarction suggesting that aspirin may have a role in modifying endothelial dysfunction.(115)

Aspirin, is there an optimal dose?

There has been enormous uncertainty about the role of aspirin initially in secondary prevention, then in primary prevention [See Table-4] and there is currently an ongoing debate as to appropriate dosage. Those advocating a low dose of aspirin in cardiovascular disease have substantiated this claim citing the effect of aspirin upon platelet cyclo-oxygenase at doses as low as 20 mg.(15) Other authorities argue that effectiveness is a function of dose versus risk factors and that there is a linear relationship between dose and risk and that the number needed to treat increases (for example from 44 to 53 in high risk category) when offset against the increased risk of major bleeding.(116)

A recent study (81) has demonstrated reduced platelet responsiveness in diabetic subjects to aspirin and that this was associated with raised HbA1c and adverse HDL and total cholesterol

concentrations. An increase in platelet turnover along with dysfibrinogenaemia, and acceleration in atherosclerosis would underline a sub-optimal platelet response to aspirin and indeed other antiplatelet agents in diabetes, thus supporting the case for a different dosing schedule according to responsiveness.

The Anti-platelet Trialists' Collaboration (69) carried out a meta-analysis of 287 trials involving anti-platelet agents. Of these, 65 trials purely involved aspirin in various doses. There was a tendency towards increased risk reduction in vascular events in the low dose (75-150 mg) group as compared with the medium (160-325mg) and high dose (500-1500mg) groups [32%, 26%, & 19% respectively]. There was only a 7% risk reduction documented in the diabetic population. There was no significant difference demonstrated in anti-thrombotic efficacy between 300mg & 1200 mg doses in the UK-TIA study,(121) while in the Aspirin and Carotid endarterectomy Study,(122) doses between 81mg and 325 mg were found to be more efficacious than the 650mg or 1500mg doses. A recent re-analysis using individual data from 6 primary prevention studies (17 000 individuals at high average risk, 43 000 person-years, 3306 serious vascular events) to compare efficacy of aspirin use long-term versus the risk of gastro-intestinal bleeding and haemorrhagic stroke concluded that the net benefit for aspirin use in primary prevention was uncertain.(68) A summary of the major trials in primary prevention of cardiovascular disease using aspirin can be found in Table-4.

Table-4: Summary of major primary prevention studies with aspirin with details of diabetes sub-population. Adapted from Raghavan R et al.,

Characteristics	BMD (117)	PHS(118)	ETDRS(80)	TPT(119)	HOT(79)	PPP(68)	WHS(123)	POPADAD(74)	JPAD study(120)
Number (n =)	**5139**	**22071**	3711	2540	18790	4495	39876	1276	2539
Number of Diabetic	103(2%)	533(2%)	3711	unknown	1501(8%)	1031(29%)	1036(2.65)	1276 (100%)	2539 (100%)
Duration of followup (in years)	5.8	5	5	6.8	3.8	3.6	10	8	4.37
Dose of aspirin	500mgs/day	325mgs alternate days	650mgs/day	75mgs/day	75mgs/day	100mgs/day	100mg alt days	100mgs/day	81-100mgs/day
Average HbA1c (% ± St Dev)	not known	not known	59% (vs 57%) of aspirin group had HbA1c<10%	not known	not known	7.6 ± 2.1	Not known	8.0±1.8	7.0±1.2%
Control	None/unblinded	Blinded placebo controlled	double blind Placebo controlled	Blinded Placebo controlled	Blinded Placebo controlled	Unblinded No placebo	blinded placebo & factorial with vitamin E	blinded 2X2 factorial with placebo and anti-oxidant	Open-label, blinded end-points Placebo controlled
Target population & risk factors	Male physicians n=101 with Diabetes (n-69, 2% in aspirin group)	Male physicians n=37 with diabetes (n=11, 4% aspirin grp)	DM(? type)-39% Definite T2DM-31% Definite T1DM-30%	Males at CHD risk Diabetes=102 (2%)	Hypertensives n-1503 (8%) with diabetes	Atleast 1 major CHD risk factor diabetes=764 (17%)	Women health professionals smoking, hypertension. Diabetes=1196	Type1 & Type 2 DM in Primary & secondary care, Smoking, BMI	Type-2 diabetes Multi-center Smoking, Hypertension, dyslipidaemia
Major/Fatal CV events Relative Risk	10% risk reduction	RR=0.34 (0.15-0.75), p<0.007	RR=0.87 (CI:0.69-1.09)	Major CV events RR=0.82 (CI:0.63-1.08)	Major CV events 15%. RR=0.85, (CI: 0.73-0.99, p=0.03)	44% 0.56(C.I. 0.31-0.99	CHD death RR=0.95 (CI:0.74-1.22, p=0.68)	non-significant, p=0.24	Composite of fatal CVD & CVA-HR= 0.1 (0.01-0.79; p= 0.0037)
Total CV Events (% risk reduction and Relative risk)	6.1%, RR=0.94 (CI=0.59-1.50, p>0.05)	Fatal+non fatal MI RR=0.56 (CI:0.45-0.7, p=0.007)	20% RR=0.80, (CI:0.57-1.11)	20% risk reduction RR=0.8 (CI:0.65 to 0.99, p=0.04)	36% all MI RR=0.64 (CI: 0.49-0.85,p=0.002)	23% 0.77(C.I. 0.62-0.95	**********	No significant difference	Overall atherosclerotic events-HR=0.8 (0.58-1.10) p=0.16
Primary End Point (s)	As above	Total CV deaths RR=0.96 (CI: 0.6-1.54, p=0.87)	All Cause Mortality RR=0.91 (CI:0.75-1.11, p=0.24)	30% reduction in all events	All MI (36% reduction), all cause mortality-7% (p=0.36)	15%	Major CV event RR=0.91 (CI:0.80-1.31, p=0.13)	Death from CHD or stroke - HR=0.98 (0.76-1.26, p=0.90)	Any atherosclerotic event, see above

Although the above studies involved high-risk populations the number of people with diabetes made up a small proportion with the number of subjects with diabetes in one study being unclear. A recent primary prevention study conducted in a Japanese population with type-2 diabetes with good glycaemic control (average Hba1c-7.1%) using 80-100mg aspirin did not show any significant reduction in overall risk from atherosclerotic events but did show benefits in the over-65 age group and in fatal coronary and cerebrovascular events.(120) This benefit for the over-65 age group was similar to that in the Women's Health Study with 26% risk reduction for cardiovascular events (123) in the aspirin group. It is clear however that the gastrointestinal side effects of aspirin are dose-dependent and this needs to be taken into consideration in any debate involving optimal dosage. With the increasing use of ACE-inhibitors, concern about a potential interaction with aspirin leading to reduced anti-hypertensive effect, has been raised.(124) However the opposite was shown by analysis from an earlier study (125) involving 11,575 patients and currently low dose aspirin (<100mg/day) is considered safe in conjunction with ACE-inhibitors. Despite convincing clinical evidence backing the COX-dependent action, the various COX-independent effects of aspirin cannot be ignored. It merits further investigation. Thus the benefits of aspirin & potential optimal dose in diabetes still remain to be clarified.

The phenomenon of aspirin resistance

Clinically aspirin resistance has been taken to represent recurrence of vascular events in subjects taking aspirin to prevent these events. Some authors have tried to establish biochemical aspirin resistance using various biochemical means of assessing platelet

aggregation (following either ex-vivo or in-vivo aspirin treatment) and bleeding time. The extent of correlation between biochemical and clinical aspirin resistance is undefined at present.

Gum et al. (126) studied 325 patients with stable cardiovascular disease using 2 methods of assessing platelet function and showed differing rates of "non-response" and "semi-response" with a tendency to increase with age and in females. The same group studied the rate of clinical events in such non-responders; follow-up showed that aspirin resistance was associated with an increased risk of death, myocardial infaraction (MI), or cerebrovascular accident (CVA) as compared with aspirin sensitivity (24% vs. 10%, HR 3.12, 95% CI 1.10 to 8.90, p = 0.03). (126) Similar findings of aspirin resistance have been noted in other studies.(127)

Definitive mechanisms for aspirin resistance are yet to be elucidated. One study of in-vitro aspirin resistance linked it to hypersensitivity of platelets to ADP.(128) Regular and concomitant ingestion of other NSAIDs seems to reduce the effectiveness of aspirin as show in a number of studies (129-131) and this may contribute towards aspirin resistance. Oxidative stress via a COX independent mechanism contributing to aspirin-insensitive TXA2 biosynthesis may also be implicated, especially in acute settings.(89) Hyperglycaemia in diabetes can cause glycation of platelets and fibrinogen,(132) as opposed to acetylation by aspirin, a process which improves platelet response and enhances fibrinolysis. In addition poor compliance with aspirin intake may be construed as aspirin "non-response".(133)

Aspirin when used for primary prevention has also been purported to be more effective in subjects with a systolic blood pressure less than 130mm Hg. (134) These and other platelet related interactions in diabetes raise questions about "clinical aspirin resistance" when looking at the non-significant primary prevention results in the diabetic population in the PPP study (70) and ETDRS study.(80)

An elevated urinary concentration of 11-dehydro thromboxane B_2 (11-DTB2) (a stable metabolite of thromboxane) was found to predict an increased risk of MI and cardiovascular death and could be a potential marker for aspirin resistance.(135) However another recent study found no correlation between fluctuations in urinary 11-DTB2 and changes in aspirin doses after cerebral infarction in the black population.(136) This may be due to smaller sample size or population specific differences.

Summary & hypothesis of study

The information presented thus far proves that aspirin despite over a century of research is still a medication offering some challenges in understanding its actions, applications, and dose-response relationships in both traditional and novel scenarios. The recent advances in understanding the pathophysiology of diabetes and cardiovascular disease, coupled with a global increase of these conditions in epidemic proportions have partly fuelled the need to exploit fully the potential of this safe, inexpensive drug. The under-utilization and mis-

medication of aspirin especially in diabetes, is a cause for concern. The recent rise in awareness of the key molecular mechanisms underlying type-2 diabetes, atherosclerosis, and the metabolic syndrome- namely oxidative stress, endothelial function, and inflammation holds promise in terms of more targeted treatment to modify disease progression.

Further research is necessary in order to resolve the appropriate dose of aspirin and whether there is a case for different doses, at different times in each specific condition that aspirin is used for. The case for such a strategy is especially strong in the diabetic population given the high cardiovascular morbidity and mortality, platelet abnormalities, dysfibrinogenaemia, and chronic inflammation, which are the hallmarks of this condition. Although studies such as CAPRIE (137) and CURE (138) have shown that clopidogrel is a more potent anti-platelet agent in the context of secondary prevention, the multitude of effects that aspirin has, the vast prescribing experience gained over the years, and its cost-effectiveness puts this drug at the centre of the therapeutic strategy in tackling the burden of cardiovascular disease in modern society. This is further borne out by the fact that several years after the advent of clopidogrel there continues to be a definite role for aspirin in vascular disease management. This may well change when clopidogrel comes off patent and more experience is gained in using this drug. Newer anti-platelet agents are also in production.

Hypothesis & Aims of study

From the above observations however the hypothesis is that aspirin may have differential effects upon novel vascular and metabolic markers which are implicated in the development and propogation of type 2 diabetes and cardiovascular disease. A further hypothesis is that lack of universal clarity with regards to aspirin use in diabetes leads to heterogeneous prescribing habits. Therefore the aims of our study specifically are-

Aims of investigation:

1. To examine current practice across a range of healthcare providers in the use of aspirin for primary and secondary prevention in patients with diabetes.

2. To determine the differential effects of aspirin titration upon vascular tone (determined by endothelial function) and inflammation, oxidative stress and insulin sensitivity in type 2 diabetic patients at high cardiovascular risk and non-diabetic subjects.

3. To examine the associations between aspirin mediated changes of vascular risk in subjects with diabetes – specifically whether these changes may be mediated through the actions of aspirin inhibition upon the activity of inflammatory markers such as Hs-CRP and sVCAM-1.

CHAPTER 2

DETAILS OF METHODOLOGY

❖ Research Design for Epidemiological study

The propose was to undertake an observational study examining the use and clinical beliefs of health care professionals involved in delivering diabetes care with respect to aspirin use.

METHODOLOGY

Examination of the use of aspirin in primary prevention was undertaken via an anonymous survey questionnaire (Survey Questionnaire can be found in Appendix-A) examining the following areas - the type of healthcare provider, their beliefs regarding aspirin prescribing in the general population, use of aspirin specifically in diabetes, any variations in practice between type 1 diabetes and type 2 diabetes and different sub-populations (e.g. those with microalbuminuria), perceived risk of using aspirin, preferred doses, concurrent prescription of proton pump inhibitors (PPI), and their views on relative and absolute contraindications for aspirin prescribing. Owing to the use of a "cascading method" of disseminating the survey link it was not possible to ascertain the number of people who received the survey who did not respond to work out a response rate.

Results were analysed for frequency of response for each question using a Likert scale. Prescribing habits across different categories of healthcare providers, the indications for aspirin use in different settings, and the influence of various patient factors on aspirin dosing were ascertained.

❖ Details of Interventional study

1) STUDY OUTLINE

In summary, a randomised double blind placebo controlled study using various doses of aspirin was carried out in 17 subjects with type 2 diabetes and high cardiovascular risk to ascertain aspirin related changes upon glycaemic control, cardiometabolic parameters, endothelial function, markers of oxidative stress, and vascular inflammation.

a) *Subjects*:

17 subjects with type 2 diabetes at high cardiovascular risk (>30% risk of a cardiovascular event within the next 10 years) (Cardiovascular risk prediction charts, British National Formulary 2004) were recruited to the study via the outpatient diabetes clinics and within Portsmouth Hospitals NHS Trust. Ethical and trial approval was obtained from the Isle of Wight, Portsmouth, and Southeast Hampshire Local research ethics committee and from the Medicines and Healthcare products Regulatory Agency (MHRA). All participants gave written informed consent. The trial was registered with the European Database for Clinical Trials in the Community (EUDRACT no-2004-001418-14) and with the International Clinical Trials Registry (ICTN no NCT00898950).

Inclusion criteria were established diet or tablet treated T2DM patients (diagnosed for a minimum of 2 years by standard WHO criteria); between the ages of 18-70 years. Exclusion criteria included age <18 or >70 yrs., established cardiovascular disease (ischaemic heart disease, cerebrovascular disease or peripheral vascular disease), warfarin use, contraindications to aspirin,

significant renal impairment (plasma creatinine>150 mmol/l), abnormal liver function, and insulin treatment.

b) Clinical protocol:

Cardiovascular screening was undertaken at initial visit with information already present on the database, medical notes, and a detailed history. A baseline blood pressure, routine physical examination, 12 lead ECG (from the Cardiology department), and medication history completed the assessment which was then used to include/exclude subjects. Cardiovascular history was updated through the course of the study to ensure that subjects were continuing to meet the baseline inclusion criteria.

Body weight was recorded using a standard electronic balance (Welch Allyn) used in the unit in a standardized manner (fasted overnight with regular clothes without shoes having cast off heavy outerwear in a fasting state), and along with height was used to calculate Body Mass Index (weight in Kg/ square of height in metres). With the subject in supine position for at least 10 minutes in the research room, an automated sphygmomanometer was used to obtain baseline blood pressures readings and all subsequent blood pressure readings. A 12 lead ECG was also performed to assess for any features of underlying cardiovascular disease and compared with previous ECGs where available to help evaluate for cardiovascular disease to ensure study requirements were being met at inclusion.

Suitable subjects were then invited to attend following an overnight fast. They had their weight and baseline blood pressure recorded at each visit as described above. Venous access was secured by means of a standard venous cannula and at the same time 35mls of blood was taken. A portion of this was sent for baseline fasting investigations including measurements of lipid parameters, serum urea and serum electrolytes and assessment of glycaemic control using HbA1c (at first

visit only) and fructosamine, using tubes pre-specified by the public health laboratory for these tests. Blood samples were also prepared for storage and later used for assays for markers of insulin resistance, endothelial function, vascular inflammation and oxidative stress using methodology described below. The following table summarises the different marker/s used to ascertain effects of different aspirin doses on the pathophysiological parameters of interest.

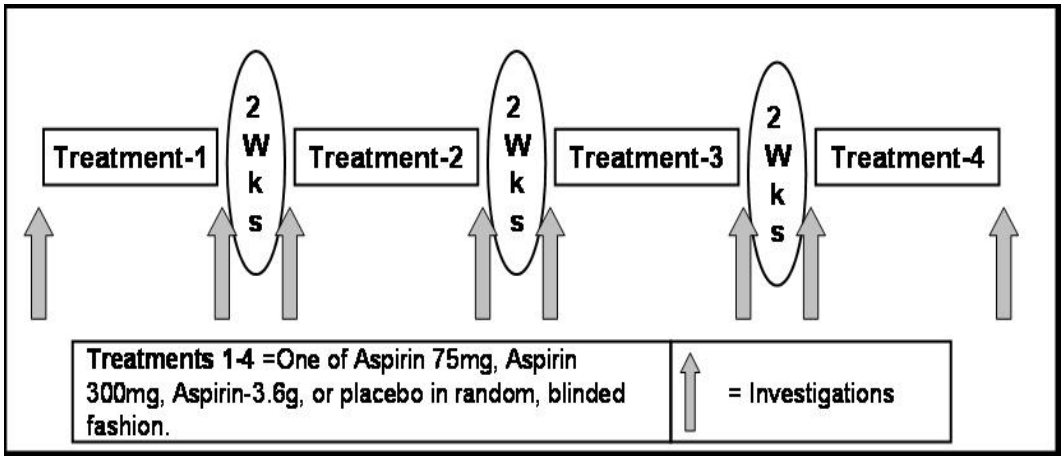
Table 5: Table of markers used

Parameter measured	Marker/method used
Metabolic markers	Blood pressure, BMI, Lipid profile, HbA1c (at baseline only), Fructosamine
Insulin resistance	Fasting Insulin and Glucose pairs used for calculations by HOMA method
Oxidative stress/ antioxidant defence	TAOS, FRAP, GSH/GSSG
Inflammation	HsCRP, S-VCAM-1
Endothelial function	Photoplethysmography

Following baseline assessment all subjects were given either aspirin (at 75mg/day, 300mg/day, 3.6g/day-generic non enteric coated, sourced through the Pharmacy Department, Queen Alexandra Hospital, Portsmouth) for 2 weeks or placebo (prepared by Pharmacy Department, St.Thomas' Hospital, London to contain standard excipients and mimic the aspirin tablets) for 2 weeks followed by 2 week washout. Following further baseline measurements the agents were then administered in a random sequence until all study subjects had crossover to each dose of aspirin and placebo, with a 2-week washout in between. The design of the study is illustrated in

figure-3. All investigations were repeated at the beginning and end of each 2-week intervention (except HbA1c) to assess response. Compliance with study medication was assessed by interview and tablet count.

Figure-3: Diagrammatic representation of flow of study.



2) MEASUREMENTS

a) METABOLIC MARKERS

Metabolic markers measured at each visit included fasting glucose, insulin (measured by Radio Immuno Assay), HOMA-IR, fructosamine, HbA1c (only at baseline visit), lipid profile (total cholesterol, HDL-cholesterol and triglycerides all by colorimetric assay), liver function tests, and urea & electrolytes.

HbA1c was measured by high performance liquid chromatography or HPLC (Menarini Diagnostics, Wokingham,UK; intra-assay coefficient of variation (CV 1.5%). Plasma total cholesterol concentration was measured by esterase and oxidase conversion (Advia 1650, Bayer

Diagnostics, Newbury, UK; CV<1.9%) and HDL-cholesterol and plasma triglyceride concentration by enzymatic determination (Advia 1650, Bayer Diagnostics, Newbury, UK; CV<1.7%)

i) Homeostasis Model Assessment

(1) Background

Calculation of the insulin sensitivity index or insulin resistance was done by the HOMA (Homeostasis Model Assessment) method after Mathews et al.(139) The gold standard for estimating insulin sensitivity has been by means of glucose-insulin clamp studies which estimate the amount of insulin and glucose required to maintain euglycaemia thus deriving an estimate of intrinsic insulin secretion. This is a time consuming and labour intensive process which is difficult to use for insulin resistance estimates in bigger studies or in the office research setting.

After Robert Turner and Rury Holman in 1976, David Matthews in 1985 published an expanded and more comprehensive structural model of estimating beta cell function (%B) and insulin sensitivity (%S) in the steady state as percentages of a normal reference population known as the Homeostasis Assessment Model (HOMA).(139) This model, written in Fortran, took greater account of peripheral glucose uptake and could use fasting levels of insulin or C-peptide in addition to insulin measurements obtained by radioimmunometric assay (RIA).(139) As an alternative to running the FORTRAN computer model, a set of linear equations were also made

available. These gave approximate values of %B and, instead of %S, HOMA IR (insulin resistance) which is the reciprocal of %S (100/%S).

The drawbacks of the HOMA equations which opened up estimation of insulin sensitivity and beta cell functioning indices to researchers conducting large scale studies included

- Not being validated for newer insulin assays.
- Not being able to account for hepatic insulin resistance and variations in insulin resistance and higher glucose levels.
- Inability to differentiate between pro-insulin and insulin.

In 1998, Jonathan Levy et al. published an updated HOMA model (HOMA2) (140) which took account of variations in hepatic and peripheral glucose resistance, increases in the insulin secretion curve for plasma glucose concentrations above 10mmol/L (180mg/dL) and the contribution of circulating proinsulin. Furthermore the model was recalibrated to provide %B and %S values of 100% in normal young adults with results from currently available assays for insulin, specific insulin or C-peptide.(140) In 2004, the HOMA Calculator was released by the Diabetes Trial Unit, University of Oxford. This provided quick and easy access to the HOMA2 model for researchers who wished to use model-derived estimates of %B and %S, rather than linear approximations.

(2) Method

Values derived from paired fasting samples for insulin (radioimmunoassay) and glucose (3 samples 5 minutes apart) were used to calculate the insulin sensitivity index (and insulin

resistance) by input into the HOMA 2nd generation calculator (141) obtained via download from the University of Oxford was used to calculate Insulin resistance. An example of the calculator interface is demonstrated in Figure 8.

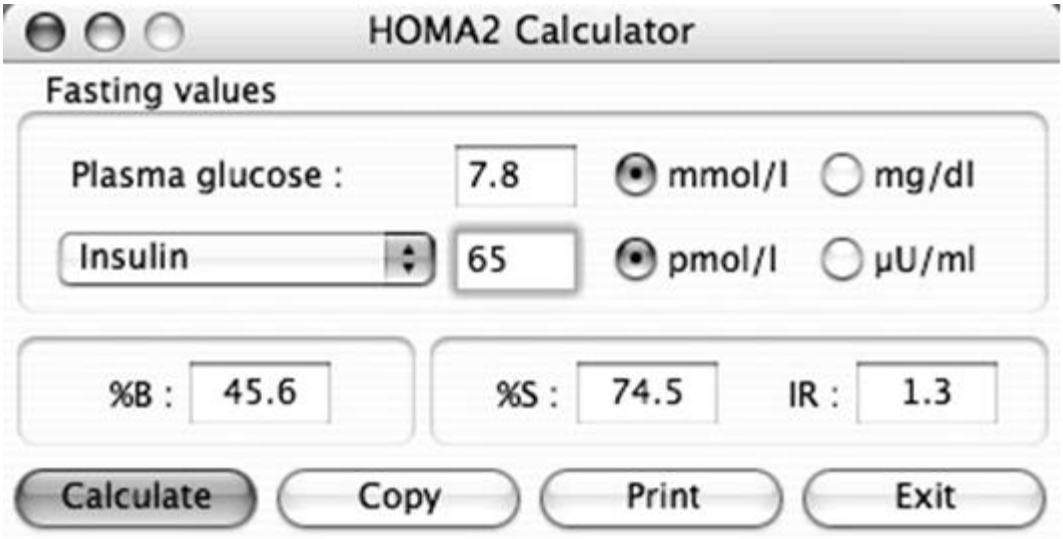


Figure-4: HOMA CALCULATOR (***Picture reproduced with kind permission from Diabetes Trials Unit, University of Oxford)***

b) MARKERS OF OXIDATIVE STRESS / ANTIOXIDANT DEFENCE

Markers included plasma total antioxidant status [TAOS] (142) (enzymatic colorimetric assay), Ferric reducing ability of plasma [FRAP] (143) (enzymatic colorimetric assay), whole blood ratio of reduced and oxidized glutathione [GSH/GSSG] (144) (enzymatic colorimetric assay).

i) Plasma Total Antioxidant Status by ABTS+ assay method (TAOS)

(1) Background

Laight et al. developed a photometric microassay (142) for the assessment of total antioxidant status in plasma at physiological pH and temperature. This assay was subsequently used to evaluate experimental oxidant stress in vivo. Plasma TAOS, expressed as the ascorbate equivalent antioxidant concentration, was found to be significantly reduced in an animal model treated with oxidant compounds for 7 days. Aorta isolated from this animal model showed impairment of endothelial function from baseline which served as a model of endothelial function in vitro.

(2) Method

2 ml blood was collected from the patient in an EDTA bottle. This was further centrifuged at 1600 x g (IEC centra- 3C, International Equipment Company, Milford, USA) for 5 minutes. The supernatant plasma was stored in a –85° C freezer based at Queen Alexandra Hospital, Portsmouth.

TAOS was assessed by the ability of plasma to inhibit the peroxidase-mediated accumulation of ABTS+ radical [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid] (142) which was an adaptation of previous assays discussed below. Generation and detection of the ABTS+ radical had previously been described in relation to assessment of total antioxidant status of food by Arnao et al.(145) This had been adapted to render it useful for the assessment of total antioxidant status of plasma by employing an inhibition assay with a fixed time point.(146)

The 100µl assay well in a 96-well plate comprised of a reaction mixture (final concentration) of 20µl ABTS (2µM, final concentration), 10µl horseradish peroxidase (30mU/ml), 10µl sample and 40µl phosphate-buffered saline (10µM PBS at pH 7.4) and 20 µl hydrogen peroxide (H_2O_2-0.1µM). The reaction was initiated by the addition of the hydrogen peroxide (H_2O_2 - 0.1µM) and conducted at 37° C. The increase in absorbance at 405 nm, reflecting the accumulation of ABTS+, was determined in a microplate reader (VERSAmax; Molecular devices Corp., Sunnyvale CA). All determinations were made in duplicate.

The intra-assay coefficient of variation (%CV) is <3% for the TAOS assay, while the inter-assay CV is <10%.

ii) Ferric reducing ability of plasma (FRAP) assay for total antioxidant capacity

(1) Background

The 'Ferric-reducing ability of plasma' (FRAP) assay was originally devised by Benzie and Strain as a novel method for assessing anti-oxidant power in 1996.(143) The automated assay described the use of antioxidants (as reductants) in a redox-linked colorimetric method, with the conversion of ferric ion to ferrous ions in a test sample being compared to other preparations with

known ferrous ion concentration. A spectrophotometer was used for absorbance measurement at 593 nm (A593) and the concentration of ferrous ion in test sample was determined. The readings were then correlated with a standard reference curve constructed with Fe II to obtain the corresponding FRAP value.

(2) Method

2.5 ml of blood was collected from the patient into a citrated tube. This was centrifuged at 1600 x g (IEC centra- 3C, International Equipment Company, Milford, USA) for 5 minutes. Supernatant plasma was then stored in the -85° C freezer. The samples thus stored were analysed by the method devised by Benzie and Strain.(143) A 300 μM acetate buffer of pH 3.6, 2,4,6-tri-(2-pyridyl)-1,3,5-triazine at 10 μM, and $FeCl_3 \cdot 6H_2O$ 20 μM were mixed together in the ratio of 10:1:1, respectively, to give the working FRAP reagent. A plasma aliquot of 50 μl was added to 1 ml of FRAP reagent in a plastic cuvette. Absorbance measurement was taken at 593 nm (A593) exactly 10 min after mixing, with 50 μl of water being used for the reference samples. Measurements were taken in duplicate at room temperature with samples protected from direct sunlight. The readings were correlated with a standard reference curve (constructed with Fe II) to obtain the corresponding FRAP (μM FeII) value. The intra-assay coefficient of variation (CV) reported in percent is <1 % for the FRAP assay, while the inter-assay CV is <3 %.

iii) Whole blood GSH/GSSG (Glutathione ratio)

(1) Background

Glutathione (GSH or γ-glutamylcysteinylglycine) is found in most cells and is the primary non-protein sulfhydryl containing molecule in aerobic oganisms. It plays a vital role in cellular defence against a variety of insults. Glutathione donates an electron during several reactions and

thus acts as a redox buffer which is important in protein synthesis, detoxification, free radical scavenging, and metabolism of xenobiotics. Changes in glutathione levels have been linked to several diseases including diabetes. Glutathione becomes oxidised (GSSG) and then through the action of Glutathione reductase and β-NADPH is returned to a reduced state (GSH).(147) Glutathione is highly unstable and is readily changed to its oxidised form (GSSG). Glutathione concentration in whole blood (as an intracellular component) is of the order of ~1mmol/L with plasma concentrations being ~1% of this. (148)

Faure et al. (144) investigated the effect of an insulin sensitiser (Metformin) on free radical activity in high fructose-fed rats, a diet that leads to insulin resistance.(144) An increase in blood levels of reduced glutathione (GSH) was noted suggesting that metformin probably had some antioxidant capacity. The ratio of reduced and oxidized glutathione (GSH/GSSG) was also used to evaluate antioxidant capacity and this was found to be elevated again suggesting that metformin had a positive influence on antioxidant function. However the significance of calculating the ratio over and above the individual components has been debated.(149)

A fall in blood glutathione level is noted in diabetes which is all the more marked when attendant complications are present.(150) An increase in GSH in isolated human red cells, treated with metformin, has been shown to improve the protection of red cell membranes against free radical damage (151) Reduced glutathione levels from subjects with diabetes showed an inverse correlation with glycaemic control in diabetes as assessed by HbA1c levels.(152)

(2) Method

2 ml of blood was collected from patients in an EDTA bottle. 1 ml ice-cold mixture of 0.5mM EDTA and SSA 10% w/v was added to this sample. This was further centrifuged at 1600 x g

(IEC centra- 3C, International Equipment Company, Milford, USA) for 5 minutes. The supernatant was then stored in cuvettes in a –85° C freezer.

Glutathione ratio was assessed using the GSSG reductase/5,5'-dithio-bis(2-nitrobenzoic acid) re-circulating method following derivatisation of GSSG with 2-vinylpyridine.(153; 154)

GSH was assessed photometrically in a plate reader at 37° C in a GSH reductase / 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) recirculating assay essentially as described by Tietze (154). The 100 µl assay well comprised (final concentration): 70µl DTNB (0.6 mM); 10µl β-NADPH (0.5 mM); 10µl sample; 10µl GSH reductase (2.5 U/ml). Reagents were dissolved in sodium phosphate buffer (120mM, pH 7.4) +6.3mM EDTA. The recirculating assay was initiated after 10 minute incubation at 37° C by the addition of GSH reductase and the initial rate determined from the absorbance increase measured every 10 seconds at 405nm over 1 minute. For the selective measurement of GSSG (=2GSH), thiols were first derivatised (1 hour) with 2-vinylpyridine (2-VP) in the presence of triethanolamine (TEA) (2µl 2-VP and 6 µl TEA added to 100µl sample). This procedure was sufficient for the full derivatisation of at least 15mM GSH.

The intra-assay CV for the glutathione assay is <3% and the inter-assay CV is <10%.

c) BIOCHEMICAL MARKERS OF INFLAMMATION/ ENDOTHELIAL ACTIVATION

Biochemical markers included highly sensitive plasma C-reactive protein (HsCRP) (ELISA) (155) and soluble Human Vascular Cell Adhesion Molecule (sVCAM-1).(63)

i) Highly sensitive C-reactive protein (HsCRP)

(1) Background

C-reactive protein (CRP) is an acute phase reactant first isolated in 1941 from the serum of a person who died of severe pneumococcal sepsis by Macleod and Avery, (156) produced predominantly by the liver.(157) There is a clear correlation with general inflammation within the body.(158) Vascular inflammation as described previously in chapter 1 is now seen as a key component of the pathogenesis of the metabolic syndrome and atherosclerosis. There is evidence to suggest links between levels of Hs-CRP and atherosclerosis (159) and that development and propagation of atherosclerosis is related to general and vascular inflammation and endothelial dysfunction. Hs-CRP is being widely used as a marker of general and vascular inflammation in population studies dealing with cardiovascular risk factors.(40; 42; 158)

(2) Method

Cardiophase HsCRP (a suspension of polysterene particles coated with mouse monoclonal antibodies to HsCRP) were mixed with sample (diluted to 1:20) and allowed to equilibriate to room temperature. The principle is that these particles get aggregated when mixed with samples covered with CRP. These aggregates scatter a beam of light passed through the sample, and the intensity of the scattered light is proportional to the concentration of the relevant protein in the sample. The mixture of HsCRP and sample were measured on the DADE BEHRING BN prospec

system analyzer. The result was further analysed by comparison with a standard of known concentration. The assigned values of CRP were standardized against the international reference preparation BCR-CRM 470.(160) The results from the analyzer were evaluated automatically and represented in mg/L. The coefficient of variation for HsCRP was 2.9%.

ii) Soluble Vascular Cell Adhesion Molecule-1 (s-VCAM-1) assay

(1) **Background –**

Human sVCAM-1 is a type 1 trans-membrane glycoprotein which has a molecular weight of 100-110 kDa and consists of 715 amino acids which typically bears seven C2-type immunoglobulin domains (161) and is thought to be induced by cytokines in endothelial cells and is important in leuckocyte recruitment as part of the inflammatory response. Soluble VCAM-1 and other adhesion molecules are implicated in a wide variety of pathological processes including chronic inflammatory conditions and atherosclerosis.(63; 162; 163)

In the Edinburgh artery study having higher levels of VCAM-1 amongst other pro-inflammatory markers (E-selectin, ICAM-1, IL-6) was predictive of progression of peripheral vascular disease and atherosclerosis as measured by the ankle-brachial pressure index (164) Circulating sVCAM-1 levels were found to be increased and NO metabolite levels reduced in subjects with hypercholesterolaemia compared to healthy controls and were reduced by vitamin-E treatment.(165)

(2) **Method –**

Samples were analysed for sVCAM-1 using the Quantikine Human sVCAM-1 assay from R&D Systems Ltd. All the samples were diluted 20 fold with calibrator diluent RD5P (made up to 100mls by diluting 20mls of the RD5P diluent into 80mls of deionised water). A dilution series was set up using the sVCAM-1 standard provided in the kit. 100μL of sVCAM-1 conjugate (monoclonal antibody to sVCAM-1 conjugated to horseradish peroxidase with preservatives) was added to each well and then 100 μL of standard and samples in duplicate were added to each well and incubated for 1.5 hrs. The wells were then aspirated and washed 4 times with wash buffer (25 fold concentrated surfactant) and 100 μL of substrate (12.5 ml of stabilized hydrogen peroxide and 12.5 ml of stabilized tetramethylbenzidine mixed within 15minutes of use and stored away from light) added to each well with further incubation for 20 minutes away from light. Then 50 μL of a stop solution of 2N sulphuric acid was added to each well with a change in colour from blue to yellow and readings taken at 450nm in a microplate reader. Results were then plotted by using the readings (minus the average zero standard optical density) on a standard curve. The final readings were then multiplied by the dilution factor of 20 to obtain sVCAM-1 values for the samples. The coefficient of variation was 2.3% for the sVCAM-1 range seen within our samples.

d) ***MEASUREMENTS OF ENDOTHELIAL FUNCTION***

i) Background

Feeling the pulse is both an art and a science passed on through the generations in both ancient and modern medical practice as an integral component of assessing well-being. The art of diagnosis by feeling the pulse is widespread among traditional healers especially in eastern medicine (e.g. China and India). A lot of value has been attached to the strength and character of the pulse and even today various pulse forms are taught as part of physical examination skills even in modern medicine (e.g. the "slow rising pulse" of significant aortic stenosis). The digital volume pulse (DVP) is a term for the peripheral pulse and its pulse waveform. Two-thirds of the resistance to blood circulation from the heart is offered by small and medium arteries,(166) where blood flow is determined by muscular tone. When blood flows along this system it produces a wave which can be traced via the peripheral pulse – Pulse wave analysis. The main inflection in such a tracing is from the initial ejection force from the left ventricle and further smaller waves peripherally are generated by the resistance offered locally by the aorta and conduit vessels, i.e. a "reflectance" back as a pressure wave which augments the forward wave. This is the so called "augmentation index". Therefore stiffness in the arteries would lead to increased resistance and increased augmentation.

Since the vasomotor response of such peripheral vessels is well correlated with that of the coronary circulation (167; 168) inference can be made of overall health of the endothelium and cardiovascular risk (167; 169) Over the years various methods have been employed in order to study arterial stiffness through information provided by the peripheral vessels in order to determine cardiovascular health.(167; 170) Resistance in peripheral vessels is partly mediated by

smooth muscle tone which is controlled through release of endothelium derived relaxing factor which is now recognized as nitric oxide (NO).(171) Therefore measurement of changes in resistance manipulated by vasoactive substances (either dependent on an intact endothelial NO system or endothelium independent NO donors) is being used to derive surrogate information of endothelial functioning.

Venous plethysmography has arguably been the most widely used method of assessing forearm blood flow and the peripheral circulation in this context. The term plethysmograph was coined in the early 19th century from the Greek term *plēthysm(ós)* increase or multiply (also (*plēthý(nein)* to increase, deriv. of *plêthos* large number) and denotes a device which for measuring and recording changes in the volume of the body or of a body part or organ. Thus plethysmography has been used to measure air in the lungs and blood flow in vessels. Venous occlusion plethysmography was first described by AJ Barnett in 1951.(172) Non-occlusive methods were subsequently described and venous strain gauge plethysmography which allows analysis of changes in forearm blood flow (FBF) in response to vasoactive substances is a well validated standard for measuring endothelial function. However it is an involved, invasive, and laborious method.

ii. Digital Volume Pulse and photoplethysmography

The DVP serves as an easy non-invasive source of information for assessing changes in the waveform under various conditions. A tracing of the DVP can be obtained by means of light refraction using a finger probe. In young healthy individuals, the DVP exhibits a clearly defined first and second peak.(173) This is thought to be a function of the waveforms generated in the

vessel by forward pressure from the left ventricle (from “heart to the finger”) causing the first peak and a reflected pressure wave (the aforementioned resistance from the periphery) causing the second wave. The timing of the reflected wave, relative to the first peak is determined by the pulse wave velocity in the aorta and conduit arteries.(174) Analysis of the waveform derived from the DVP can help elicit information about vascular tone and indirectly the endothelium which is an essential part of the cardiovascular system. The maintenance of the contour of the pulse waveform with only changes in amplitude caused by factors such as respiratory function, temperature, and sympathetic nervous system activity, make this a good index to study.

Millasseau et al. (170) showed that the relationship between the DVP and the radial pressure pulse (or the digital pressure pulse) could be represented by a single mathematical transfer function. This would suggest that the information in the DVP along with the physiological determinants is similar to those of the radial pressure pulse.(170) A reflection index (RI) can be defined as the ratio of the reflected wave to the first peak (Figure 5). Millasseau proposed that the time between the first peak and the second peak (peak to peak time) be used as a surrogate measure of pulse wave velocity and arterial stiffness.(170) In older individuals, the second peak of the DVP can be attenuated and replaced by a point of inflection in the down slope of the waveform.(173) This inflection point can be used to determine the reflection index.(170) Changes in the RI in response to vasoactive drugs have been shown to mirror changes in the radial or aortic pulse.(175)

iii) Endothelium dependent and independent vasodilators:

Endothelium independent vasodilators such as glyceryltrinitrate (GTN) have been used to assess the effect of an intervention independent of any endothelial interaction. A subsequent use of an

endothelium-dependent vasodilator, which would be a trigger for the release of nitric oxide from the endothelium, could be useful in assessing the integrity of the endothelium and thereby the effect of any intervention on endothelial function.(176) In this study, based upon this principle, GTN has been used as an endothelium independent vasodilator and salbutamol as an endothelium-dependent vasodilator.(177)

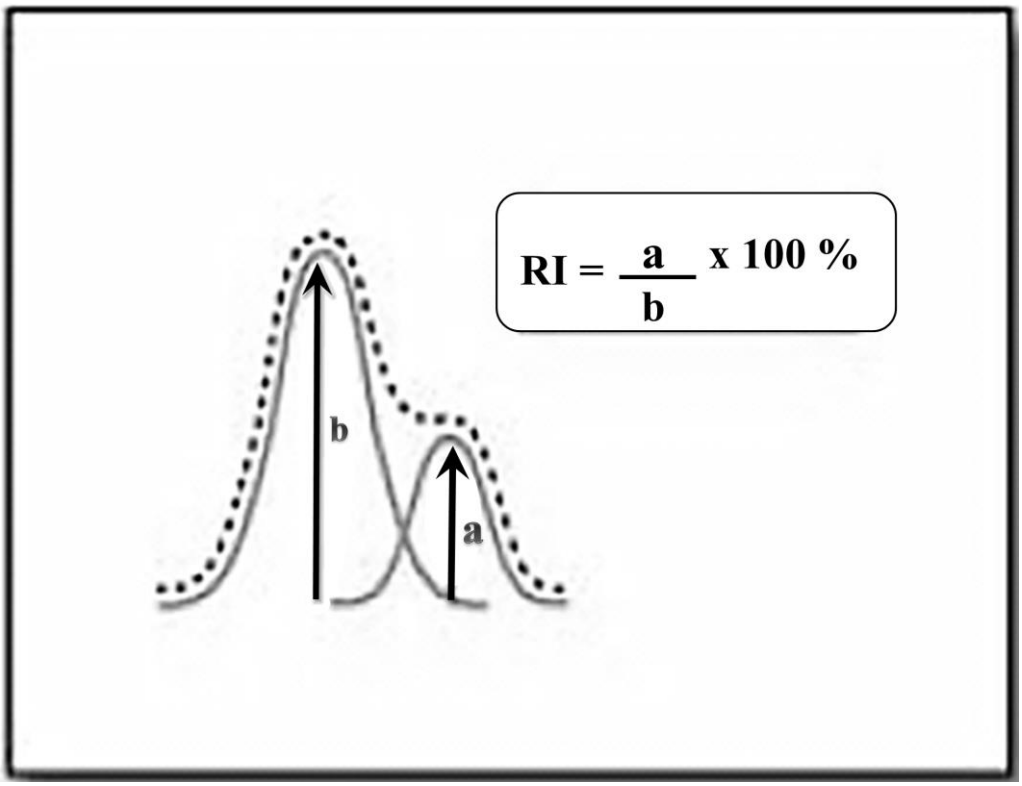


Figure 5: Calculation of the Reflection Index (RI)

(Picture reproduced with kind permission from Micromedical Ltd)

Figure 6: Technique of photoplethysmography

(Picture reproduced with kind permission from Micromedical Ltd)

Salbutamol activates β_2 adrenergic receptors on endothelial cells and produces vasodilation, partially through the release of nitric oxide by the endothelium.(178; 179) The sensitivity of the Reflection Index (RI) to exogenously administered nitric oxide donors suggests that, with the aid of a suitable stimulus which would trigger the release of nitric oxide from the endothelium of the systemic vasculature, the RI could be used to isolate measurement of endothelial function from the vasodilator activity of vascular smooth muscle.(170)

ii) Technique:

Endothelial function was measured using a photoplethysmograph (Micro Medical Pulse Trace, Rochester, Kent, UK) using a technique available within our laboratory. Each subject was placed in the supine position for 20 minutes in a quiet regulated environment, with the probe placed upon the index finger (Figure 6). Following this rest phase, measurements were taken at baseline, following administration of sublingual GTN (an endothelium-independent vasodilator) and following inhaled salbutamol (an endothelium dependent vasodilator) to determine the digital volume waveform [DVW].

Three readings were taken at baseline (average taken for reporting purposes) and then 400mcg of sublingual GTN was administered. Readings were taken at 3 and 5 minutes. A washout period of 20 minutes was allowed after which another reading was taken to confirm the return to baseline. Inhaled Salbutamol (400 mcg) was administered using a standardised technique via a spacer device, and readings taken at 10, 12 and 15 minutes. Average of the last 2 readings was taken for reporting purposes.

iii) Interpretation:

From the DVW, the reflection index (the second derivative of the pulse wave form) expressed as a percentage of the 2nd peak to the 1st waveform peak represents vascular tone in the small/medium arteries and has been validated in subjects with type 2 diabetes and in healthy volunteers.(177) The change from baseline (Δ) in reflectance index (RI) of the digital volume pulse following GTN (average of readings 3 and 5 mins post-administration) and salbutamol (average of readings 12 & 15 minutes post administration) were taken to represent endothelium independent and dependent vasodilatory functioning respectively.

STATISTICS:

Statistical analysis:

Statistical software Graphpad Instat and XLStat 2007 were used for statistical analysis.

Repeated measures Analysis of Variance (ANOVA) were used to compare baseline and interventional data as appropriate. ANOVA was undertaken with the Bonferroni post-test if the distribution was parametric, while Friedman's test with Dunn's post-test were used if the distribution was non-parametric. The Kolmogorov-Smirnov test (KS test) was used to assess whether distributions were parametric or non-parametric.

Normally distributed data, are expressed as mean ± standard deviation (SD) while the non-parametrically distributed data are shown as median&interquartile range.

Multiple linear regression analysis was used to determine the associations between baseline and intervention mediated changes in each of the variables measured using non-parametric method. P value <0.05 was considered to be significant.

It was determined that a sample size of 17 would allow the detection of a 20% difference in markers of oxidative stress with 80% power based on aspirin use in a previous study.(104) For the observational study summary statistics were prepared and contingency tables were used and Pearson's chi-square test was used to analyse the responses to survey questions. Odds ratios were worked out for those survey questions that threw up statistically significant differences.

CHAPTER 3: RESULTS

Summary of the results from both components of the study are presented at the beginning of this body of work.

Section 1 – Survey of Aspirin use in Diabetes- Detailed Results

a) Demographics of respondents:

The survey was undertaken by healthcare professionals from the south of England working in primary and secondary care and predominantly included doctors and nurses who were involved in diabetes care (n=149/152, 98%). General Practitioners and doctors in secondary care (i.e. practising within a hospital setting) made up for 59/152 of the respondents (38.8%). Diabetes specialist nurses in primary (n=69/152, 45.4%) and secondary care (n=22/152, 14.4%) along with made up a majority of the remainder of the respondents. The breakdown of numbers is graphically represented in figures 7 a-c below.

Within secondary care, sub-consultant grades made up for 7.89 % (12/152) of the survey. The majority of secondary care doctors were practising in diabetes (32/34, 94 %).

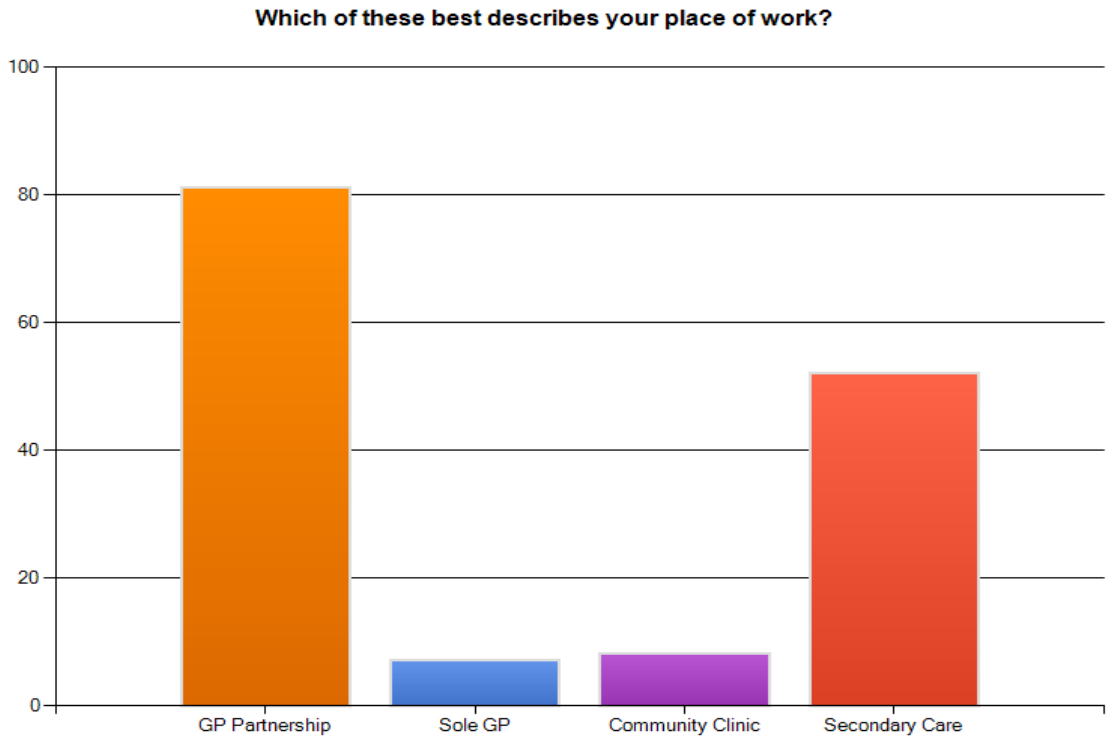


Figure-7a: Breakdown of survey respondents by place or work

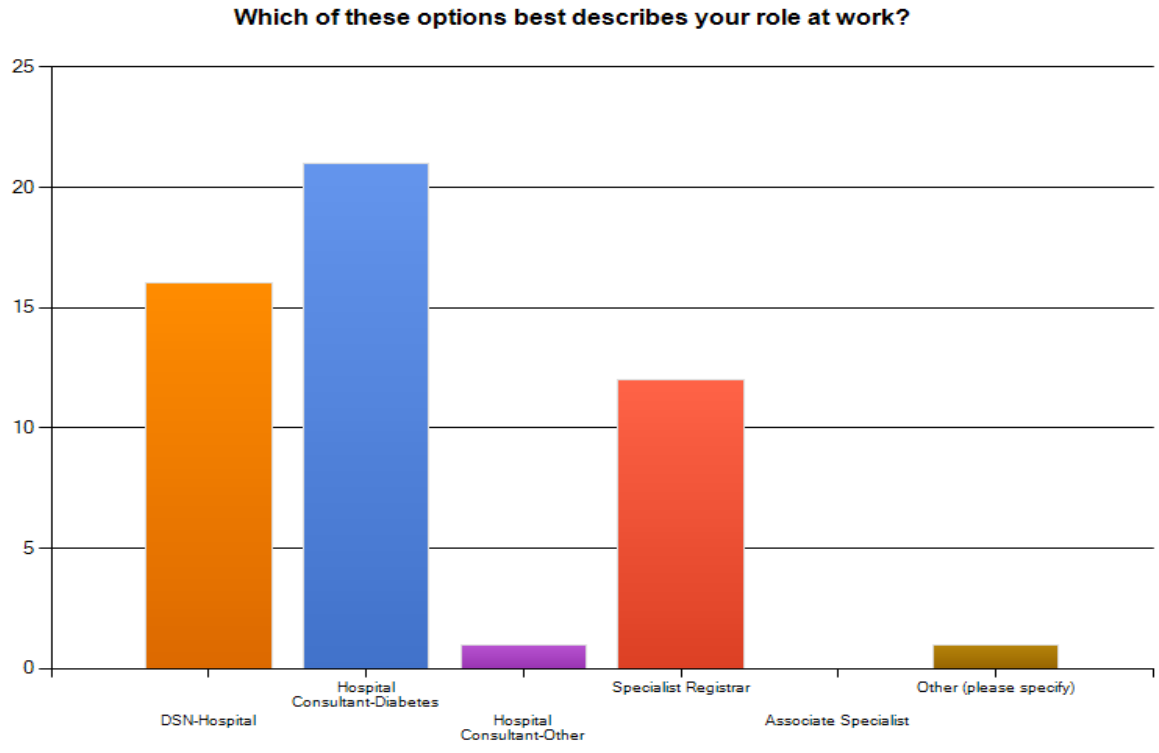


Figure-7b: Breakdown of survey respondents by role at work

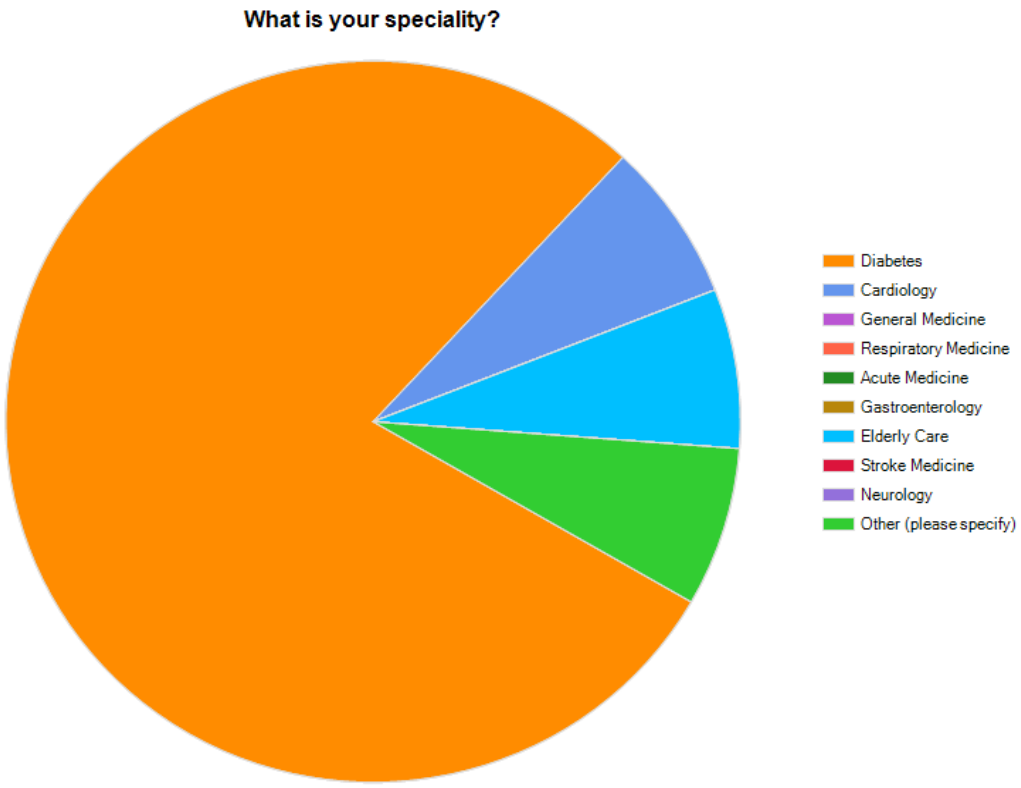


Figure-7c: Breakdown of secondary care doctors by speciality

b) Results for secondary Prevention and aspirin use:

In this survey 42.7% and 21.9% of primary care respondents felt that they either "strongly agreed" or "agreed" that aspirin should be prescribed in 'everyone as secondary prevention unless there were contraindications'. Similar numbers were adduced for primary care respondents within the individual categories for secondary prevention (19.8 to 25 % agreed and 33.6 to 46.7% strongly agreed), see figure-8a. There was stronger agreement for aspirin use in secondary care as noted in figure-8b. Thus secondary care respondents prescribed aspirin more often (91.9% vs 84.9%) to "everyone as secondary prevention barring a contraindication" as compared to their primary care colleagues (excluding non-responses) but the difference was not statistically significant (OR=1.68, 95% CI:0.49-5.96; p-value>0.05, 2-tailed). Doctors (both in primary and

secondary care) were more likely to choose aspirin in this setting compared to nurses (59/67 [88.05%] vs 51/85 [60%], OR-3.33 95% CI 1.27-8.92, p=0.008). Total figures for the use of aspirin in secondary prevention are outlined in figure-8c.

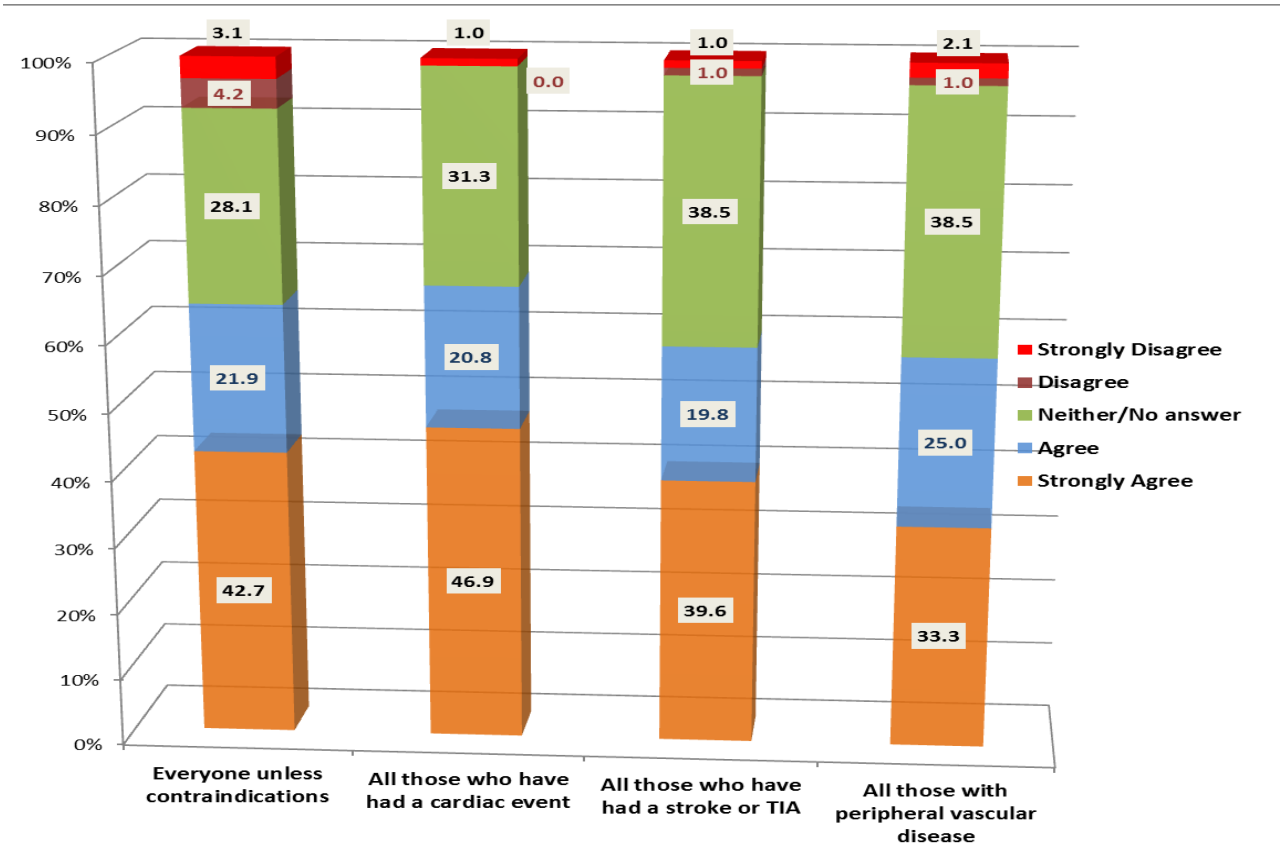


Figure-8a: Response details for "Aspirin use in secondary prevention" in Primary care

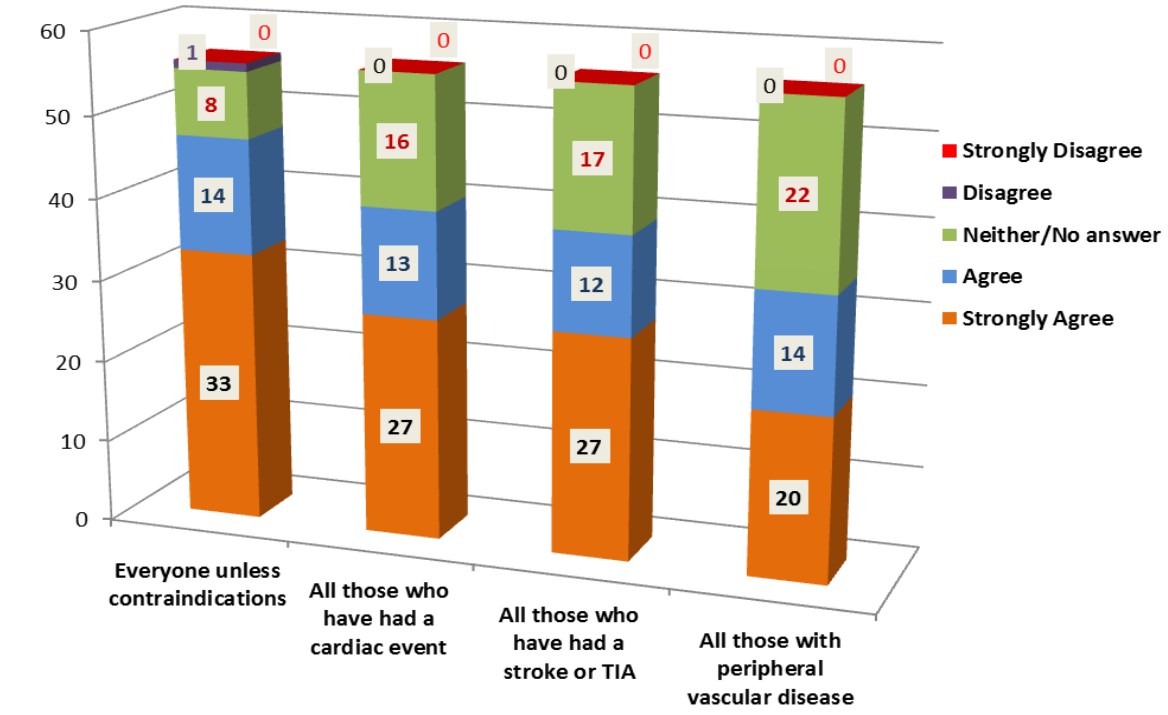


Figure-8b: Response details for "Aspirin use in secondary prevention" in Secondary care

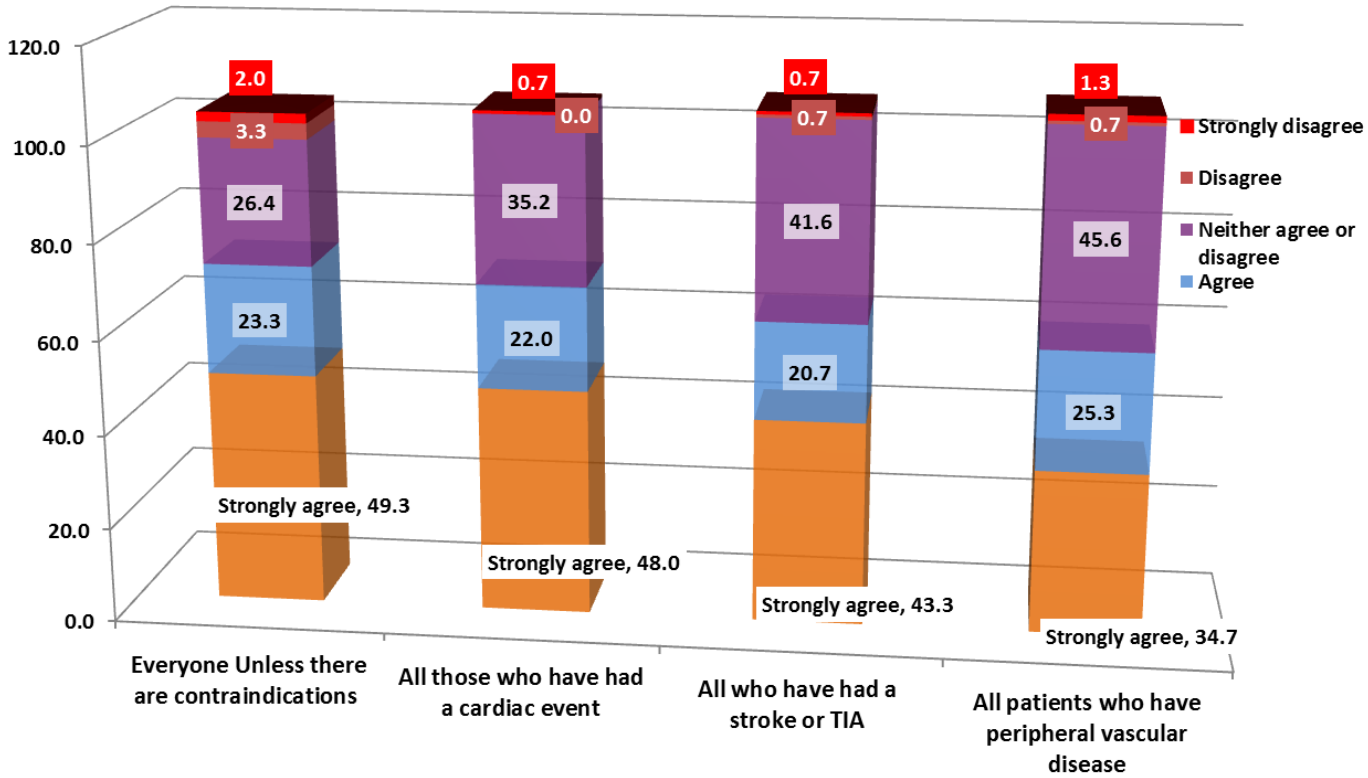


Figure-8c: Overall Response details for "Aspirin use in secondary prevention"

Only 6.9% of the respondents were not willing to use anti-platelets when confronted with patients who had a history of peptic ulceration in the context of secondary prevention. A further 22.8% opted to use an alternative anti-platelet agent (see figure-9).

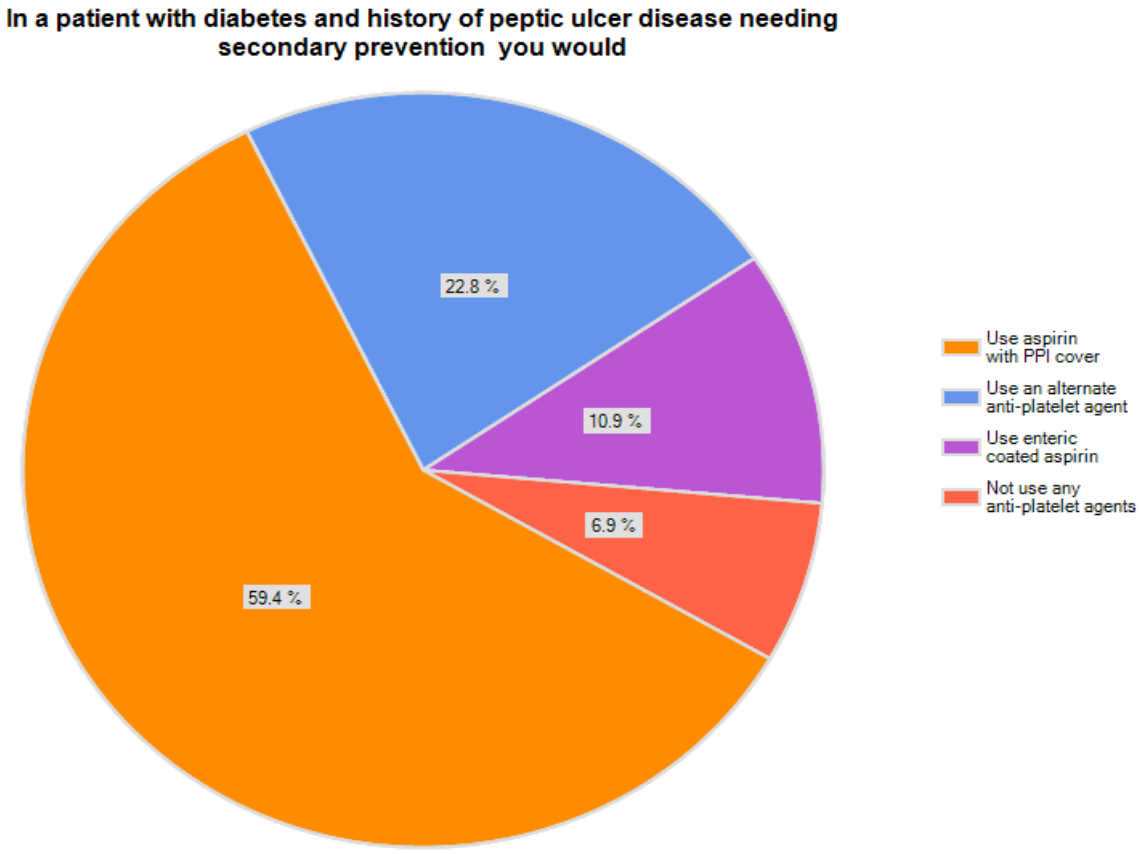


Figure-9: GI protection strategies for subjects with diabetes needing aspirin as secondary prevention

c) Results for Primary Prevention and aspirin use:

Primary prevention refers to the use of treatment that aims to 'prevent' an event from occurring when there are pre-existing risk factors. There has been enormous controversy about the use of

aspirin in this sphere especially in diabetes. There is varying guidance about the use of aspirin in primary prevention in diabetes. This confusion was reflected in our survey.

Overall only 10.7% would give aspirin in primary prevention but 47.9% were undecided. These numbers were similar when broken down into those in primary and secondary care (see figures 10 a-c). These figures are somewhat less ambiguous when broken down by sub-categories of risk especially in secondary care. Thus 34/56 (60.7%) of respondents were in favour of using aspirin in those with > 20% risk of cardiovascular events. 38% in primary care were of a similar opinion in this category. Similar comparisons for 'strong family history of heart disease' were: secondary care - 51.8% (29/56) in favour of using aspirin, primary care – 40.6%, and overall – 45.3%. Other categories such as smoking history and co-existing hypertension elicited lesser approval.

Hypertension: primary care - 29.4%, secondary care – 48.97%, overall – 29.4% approval. (20/68 vs 24/49; OR=2.3, 95%CI: 1-5.99, p=0.03)

Smoking history: primary care – 22%, secondary care – 37.5%, overall – 22% approval.

(15/68 vs 18/48, p=0.06)

Hyperlidaemia: primary care – 34.3%, secondary care – 38.8%, overall – 28% approval. (23/67 vs 19/49, p>0.05)

Microalbuminuria: primary care – 22.3%, secondary care – 43.75%, overall – 24% (15/67 vs 21/48; OR=2.69, 95%CI: 1.12-6.58, p=0.02)

These results seem to indicate a difference in risk perception between primary and secondary care in diabetes.

Further analysis demonstrated that there was significant difference in prescribing attitudes between doctors and nurses, as set out in table-6 below, which may account for some or all of the differences in prescribing attitudes between primary and secondary care.

Table-6: Summary of differences in aspirin prescribing attitudes between doctors and nurses for different survey questions.

Survey Question	Doctors	Nurses	Pearson's chi-Sq	p-value-ChiSq	odds ratio	OddsRatio P-value 2-tailed
Aspirin in primary prevention	21/54=N	18/49=N	0.05	0.8	Not done	Not done
Aspirin in all as 2ndry prevn unless C/I	59/67=Y	51/85=Y	7.511	0.006	3.33 (1.27-8.92)	0.008
Everyone prim prevention	5/59=Y	11/63=Y	2.15	0.14	Not done	Not done
Smoking+Prim Prevn	22/60=Y	11/57=Y	4.35	0.037	2.42 (0.97-6.13)	0.042
VascRisk>20%+Prim Prevn	40/60=Y	31/60=Y	2.79	0.09	Not done	Not done
Htn+Prim Prevn	38/60=Y	16/58=Y	15.18	0.0009	4.53 (1.98-10.71)	0.0009
Hyperlipidaemia+Prim Prevn	26/60=Y	16/57=Y	2.96	0.08	Not done	Not done
Microalbuminuria+Prim Prev	26/60=Y	11/59=Y	8.464	0.004	3.34 (1.35-8.36)	0.005
One dose for all	47/56=Y	32/49=Y	4.865	0.027	2.77 (1.01-7.77)	0.041
PPI+Enteric s other-2ndry prev	48/57=Y	23/46=Y	13.912	0.0009	5.33 (1.96-14.88)	0.0009
PPI+Enteric s other-Primary prev	32/63=Y	18/48=Y	1.945	0.16	Not done	Not done
Routine PPI use	5/57=Y	9/46=Y	2.52	0.11	Not done	Not done

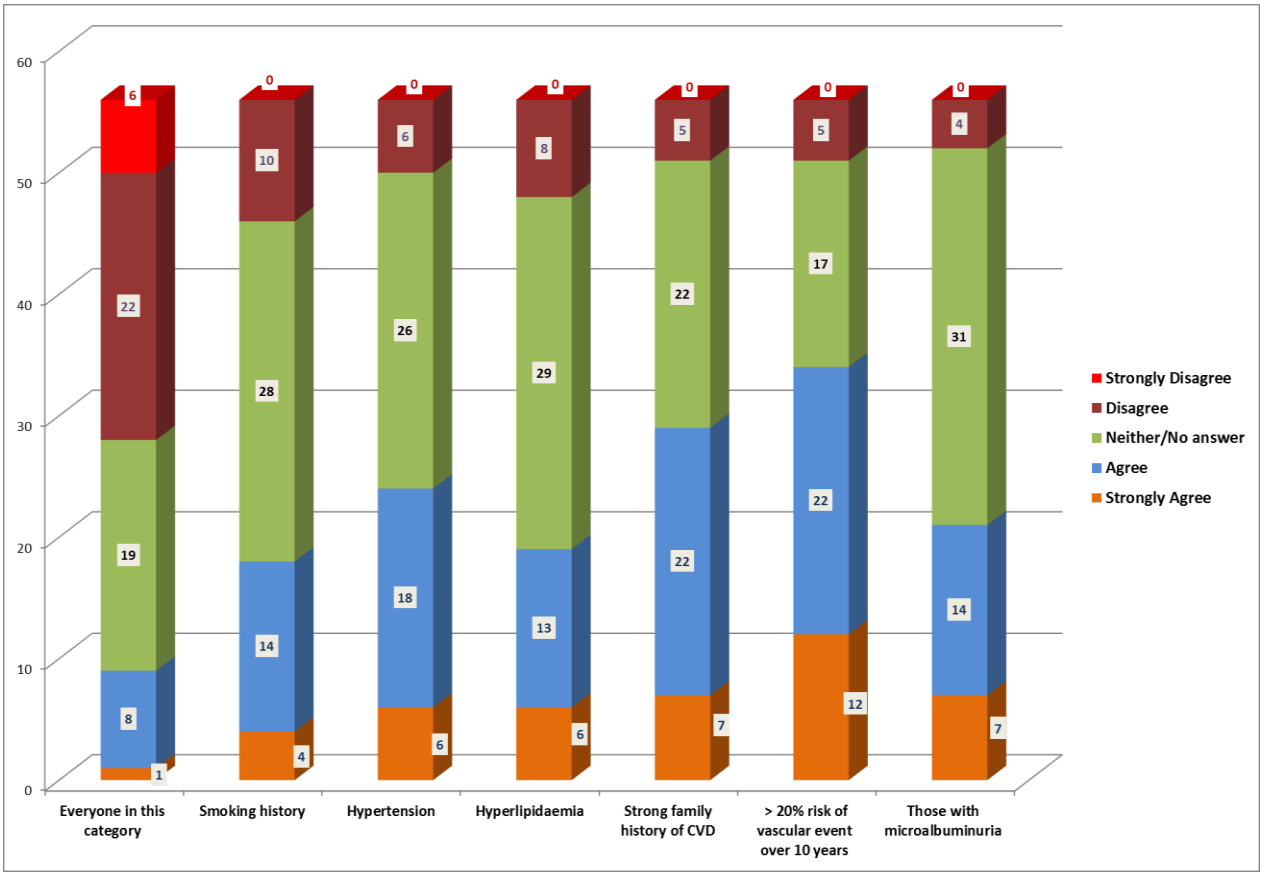


Figure-10a: Response details for "Aspirin use in Primary prevention" in Secondary care

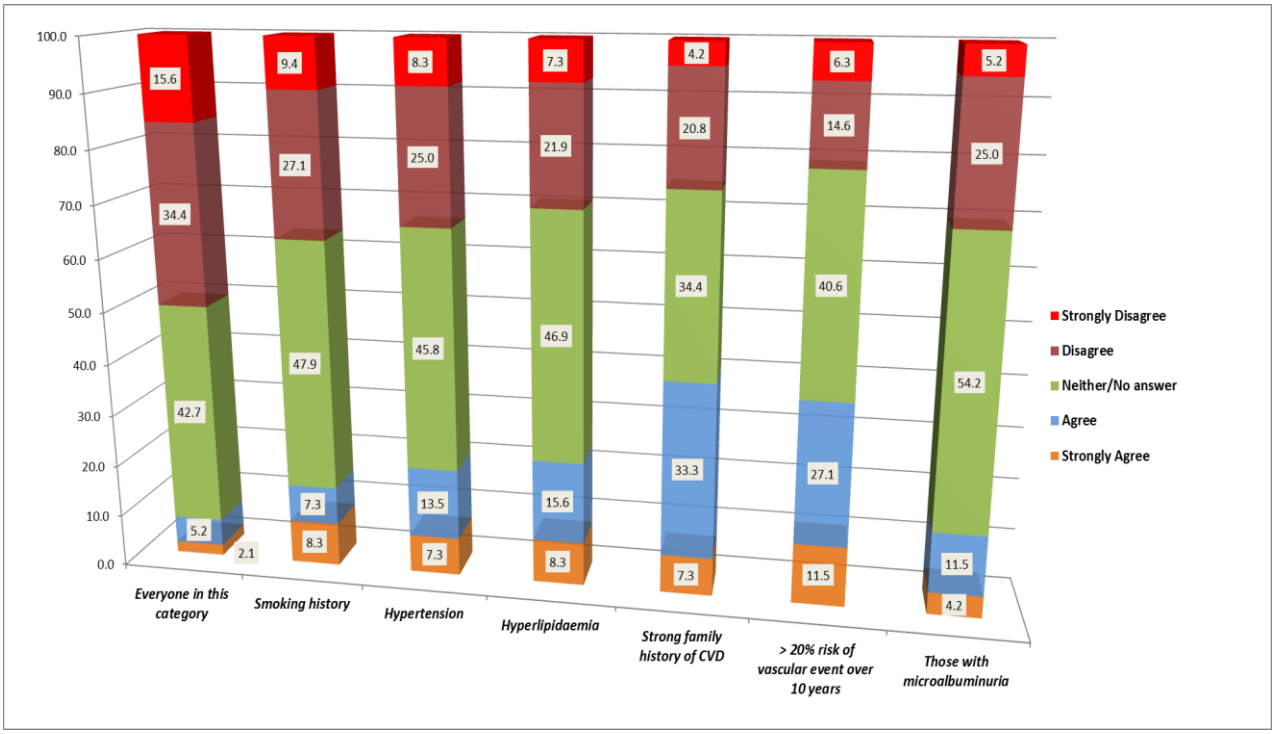


Figure-10b: Response details for "Aspirin use in Primary prevention" in Primary care

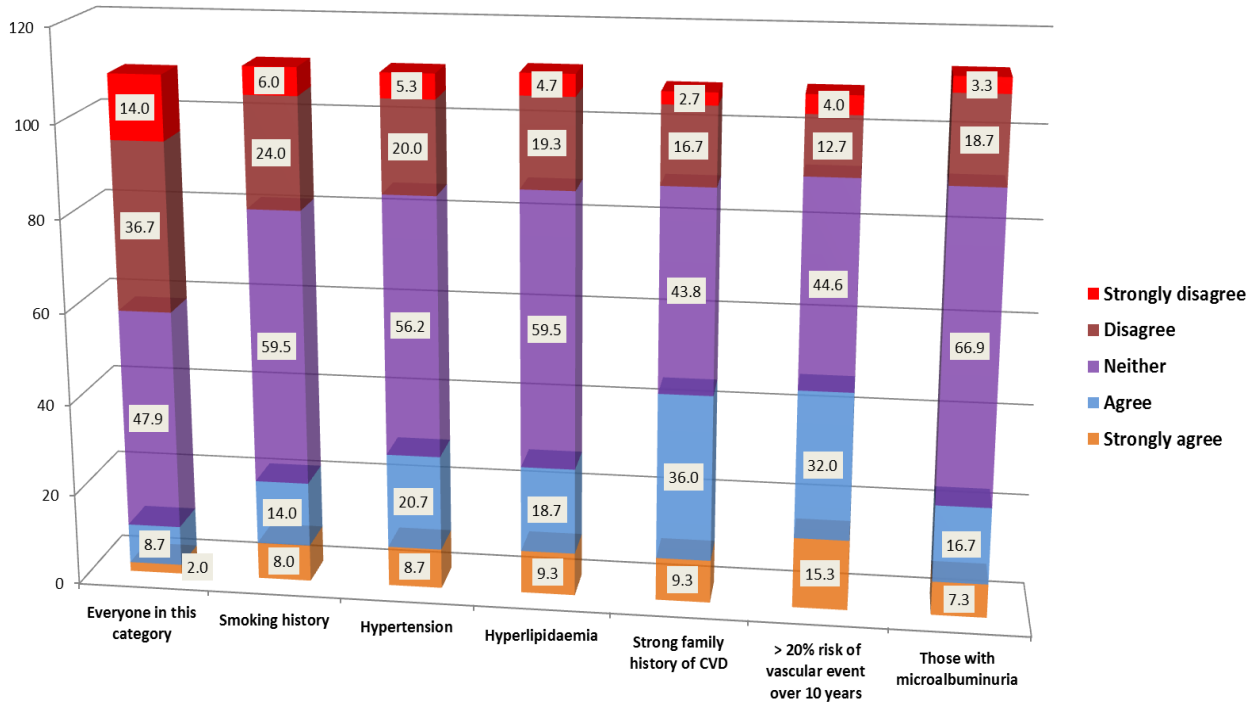


Figure-10c: Overall response details for "Aspirin use in Primary prevention"

d) Results on dose range of aspirin:

Study of differences in efficacy of various doses of aspirin and whether there should be differential aspirin dosing in different settings remains important. Given that currently aspirin 30 to 600 mg is recommended post thrombotic stroke and myocardial infarct either as a stat dose or for a fixed duration (300mg for 2 weeks post stroke) there is already a precedent for higher doses being used in certain situations. This is despite clear evidence that antiplatelet effects of aspirin are well established even at doses as low as 37.5mg.(180) In our survey respondents were mostly in favour of a single dose of aspirin (78/104, 75%). 10.6% were undecided and 14.4% felt that a single dose would not suffice (see figure-12). If we however include the 48 people that didn't

answer this question and categorise them as being "undecided" then the results are far less clear-cut.

A majority of the respondents felt that type 1 and type 2 diabetes should be considered similar in respect of aspirin use (see figure 11 and 12). Moving onto aspirin doses in specific settings as set out in figures 13a-d, 75mg/day was the commonest dose recommended. However as discussed previously there are guidelines for using higher doses in the acute setting and the survey responses seem to vary with a higher proportion of respondents recommending a lower dose when a higher dose is recommended. A small percentage of respondents recommended aspirin 150mg/day in secondary prevention (see figure-13c).

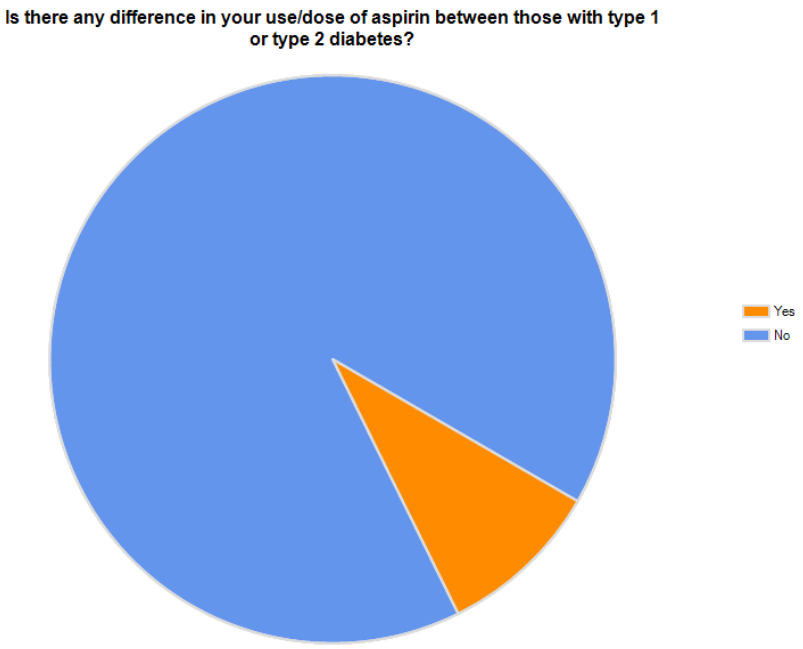


Figure-11: Aspirin usage in type 1 and type 2 diabetes.

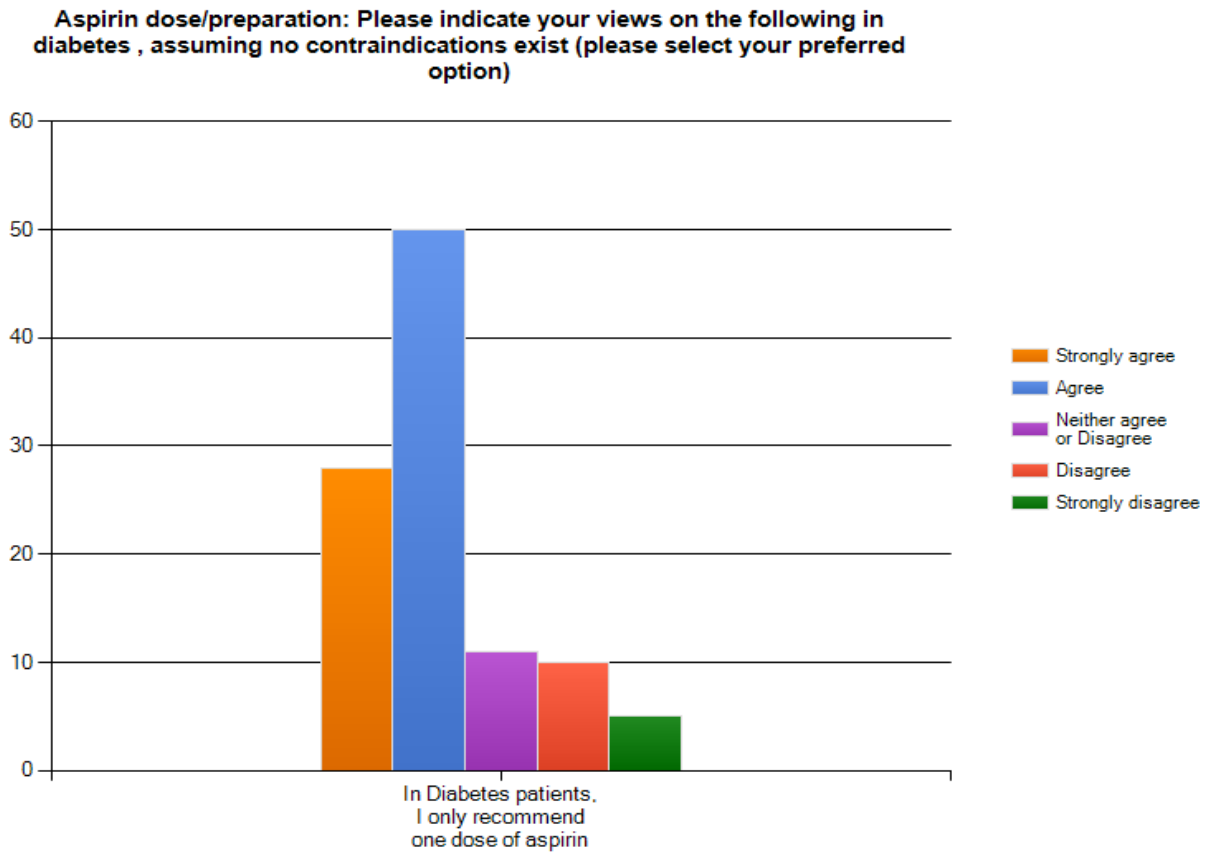


Figure-12a: Overall results of question on uniformity of dose of aspirin used

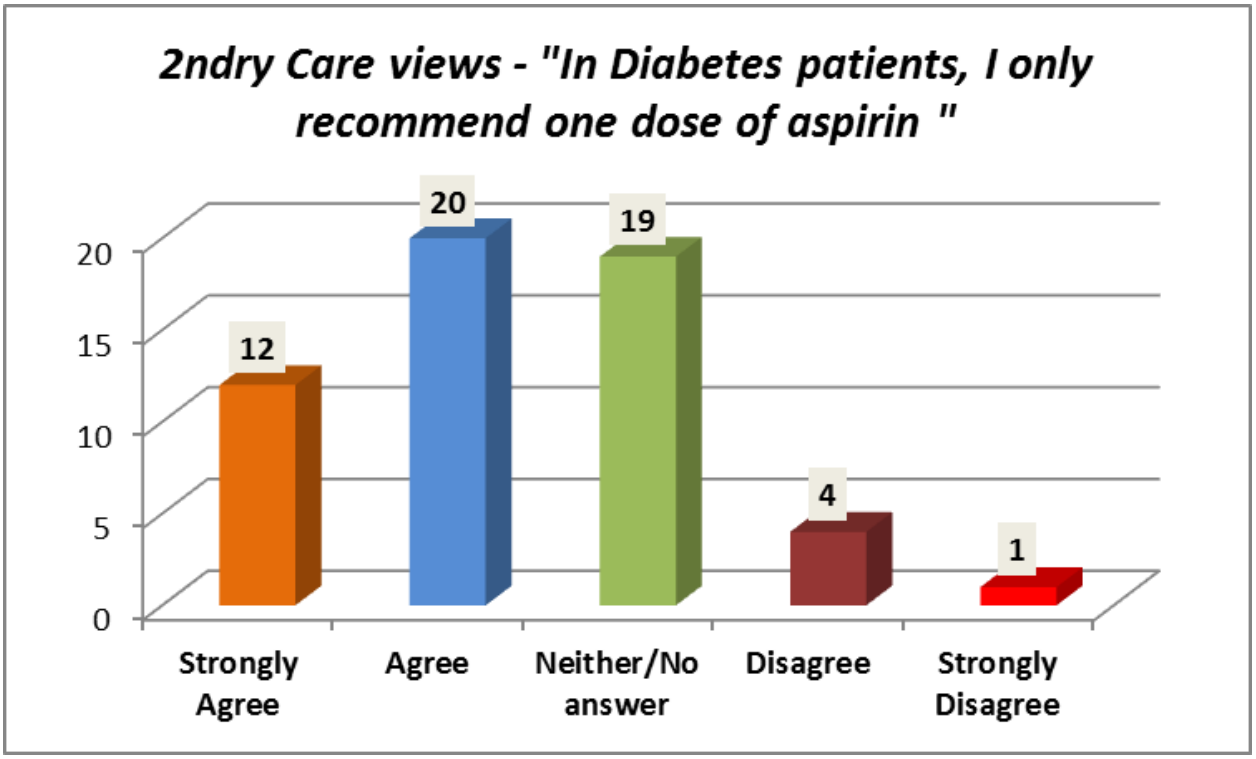


Figure-12b: Secondary Care results of question on uniformity of dose of aspirin used

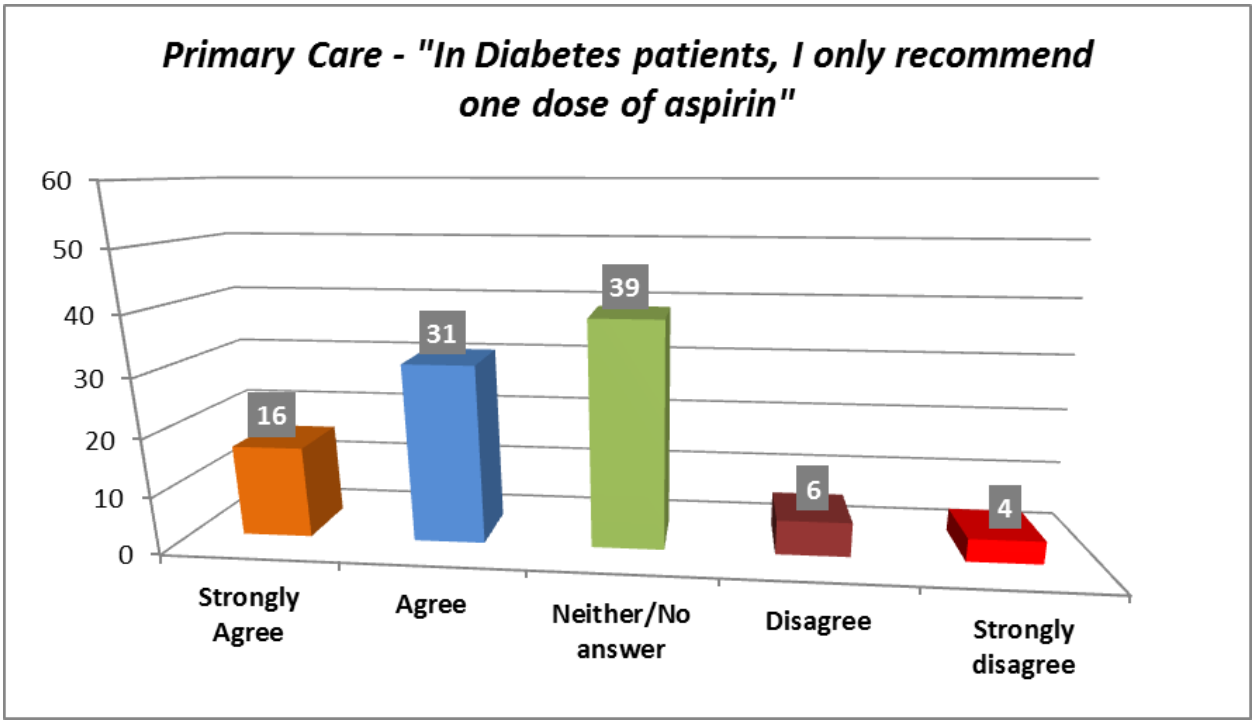


Figure-12c Primary Care results of question on uniformity of dose of aspirin used

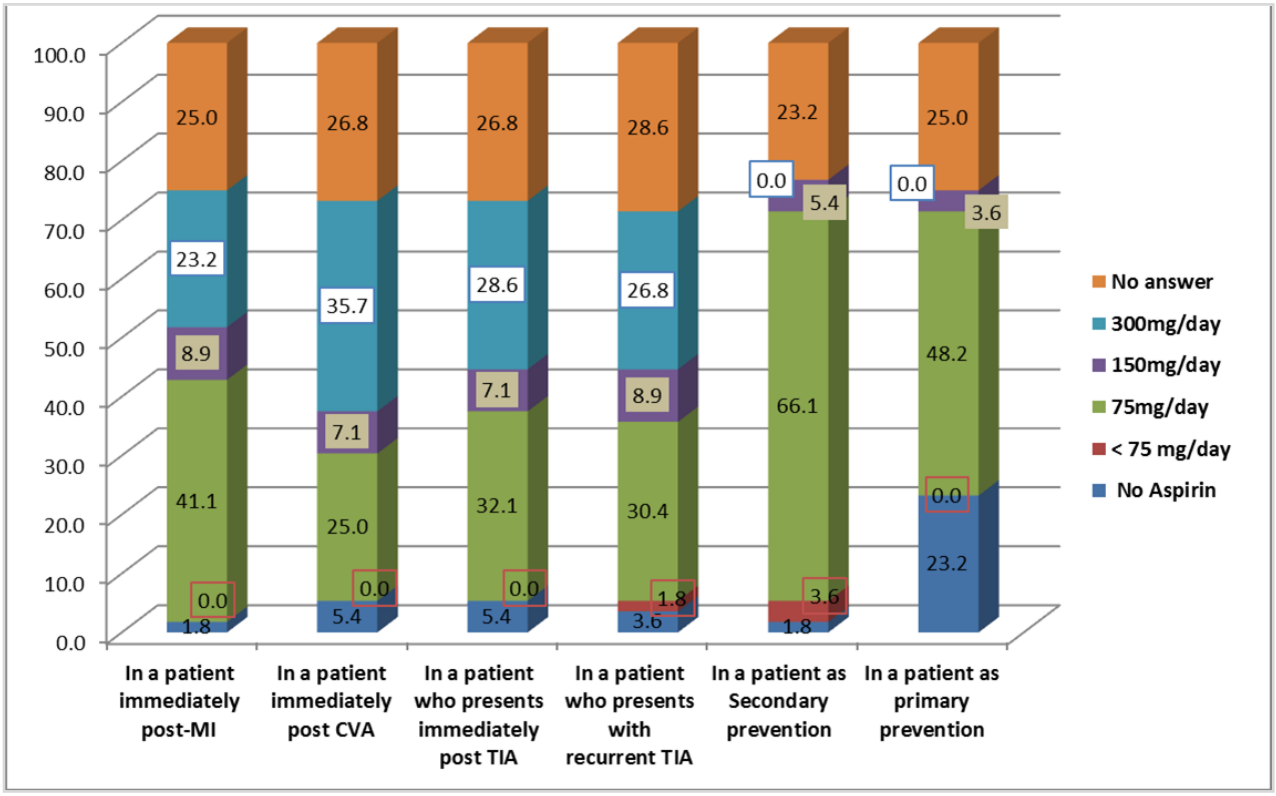


Figure-13a: Summary of responses from secondary care for dose ranges of aspirin in various settings

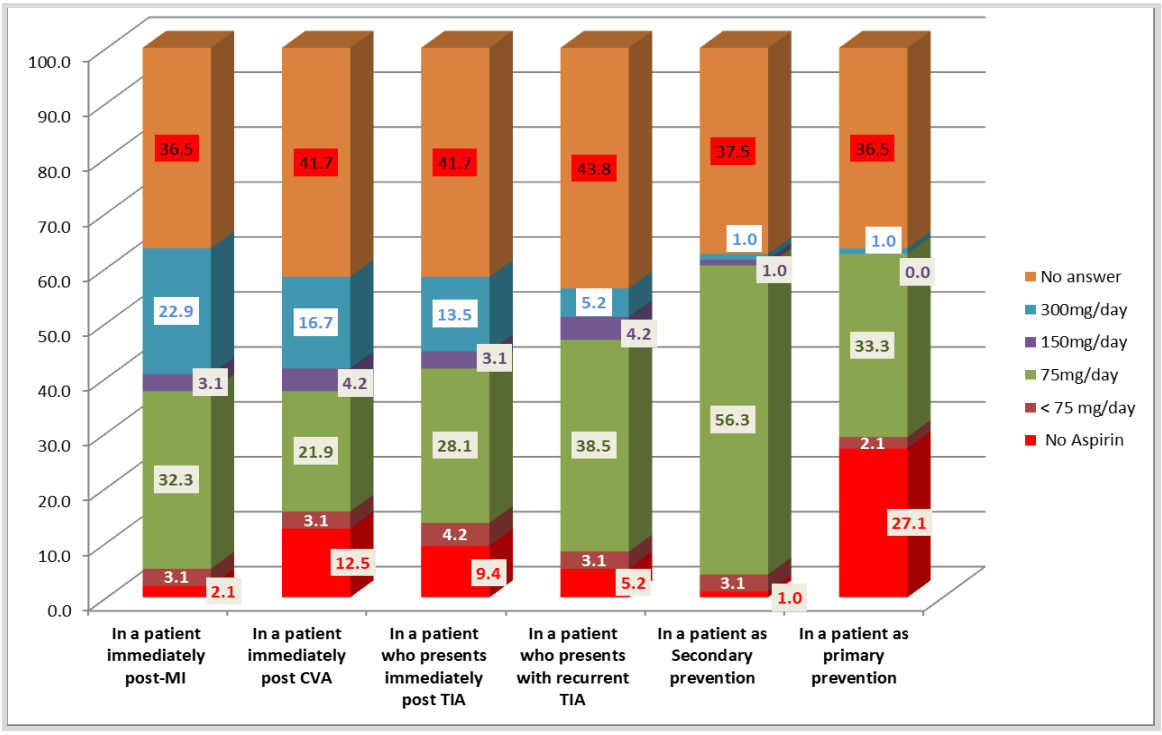


Figure-13b: Summary of responses from Primary care for dose ranges of aspirin in various settings

e) Results of other questions:

Only 13.9% of respondents routinely prescribe PPIs when giving aspirin (see figure 14 below). There is very little co-prescription of antacids along with aspirin. Enteric coated aspirin was used routinely by 7.5% of respondents and more than a third did not use any (see figure 15). There were no statistically significant differences between primary care and secondary care with regards to aspirin use with PPI or as an enteric coated version in those with history of peptic ulceration and secondary prevention (42/59 vs 29/44, $p>0.05$). However doctors were more likely (compared to nurses) to opt for continued aspirin use, as opposed to alternative anti-platelets or eschewing all anti-platelet therapy (48/57 [84.2%] vs 23/46 [50%], OR=5.33, 95%CI:1.96-14.88, $p=0.0009$) in this setting. There were no significant differences between any of these groups for aspirin use in those with history of peptic ulceration in the primary prevention setting.

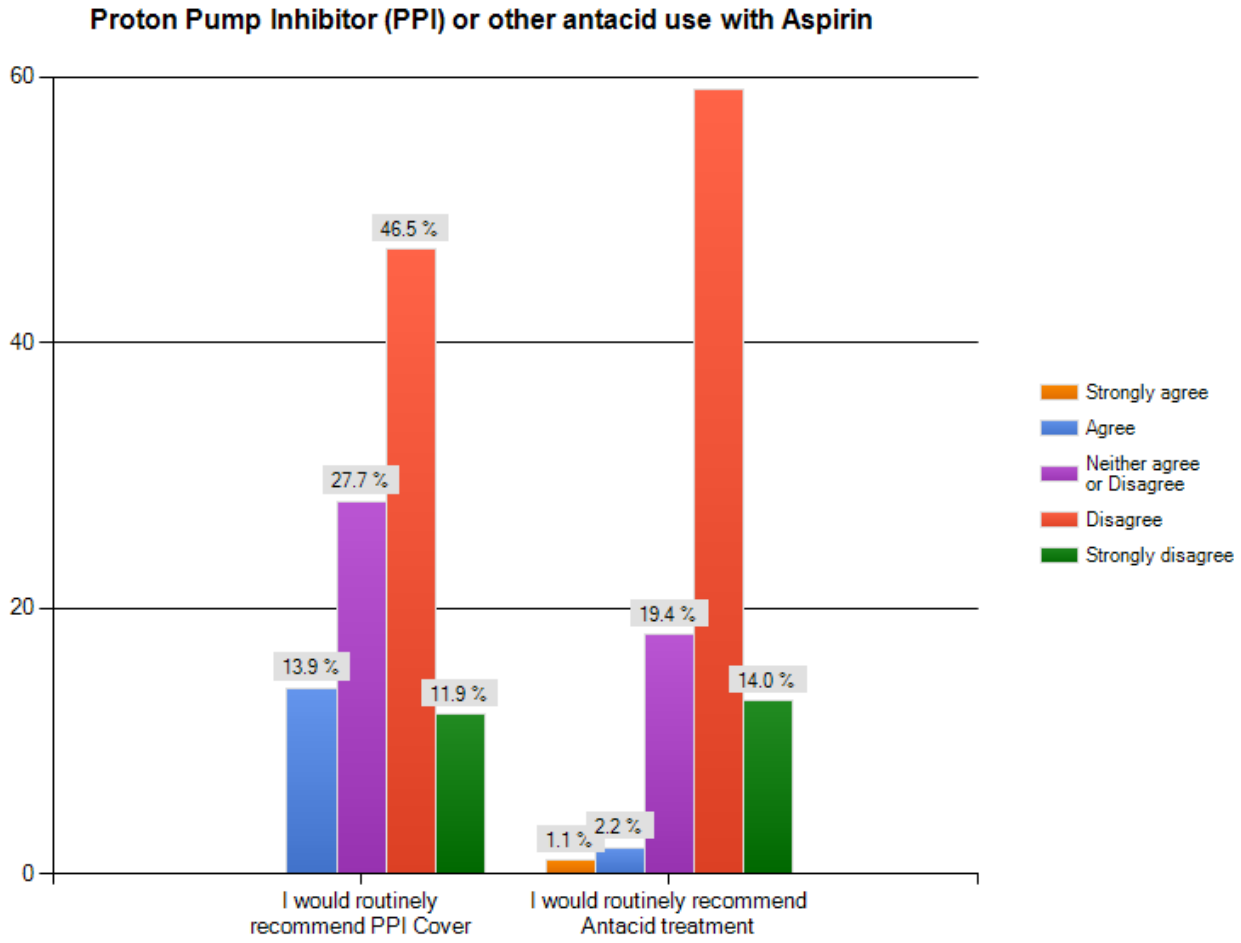


Figure-14: Use of gastric protection strategies alongside aspirin.

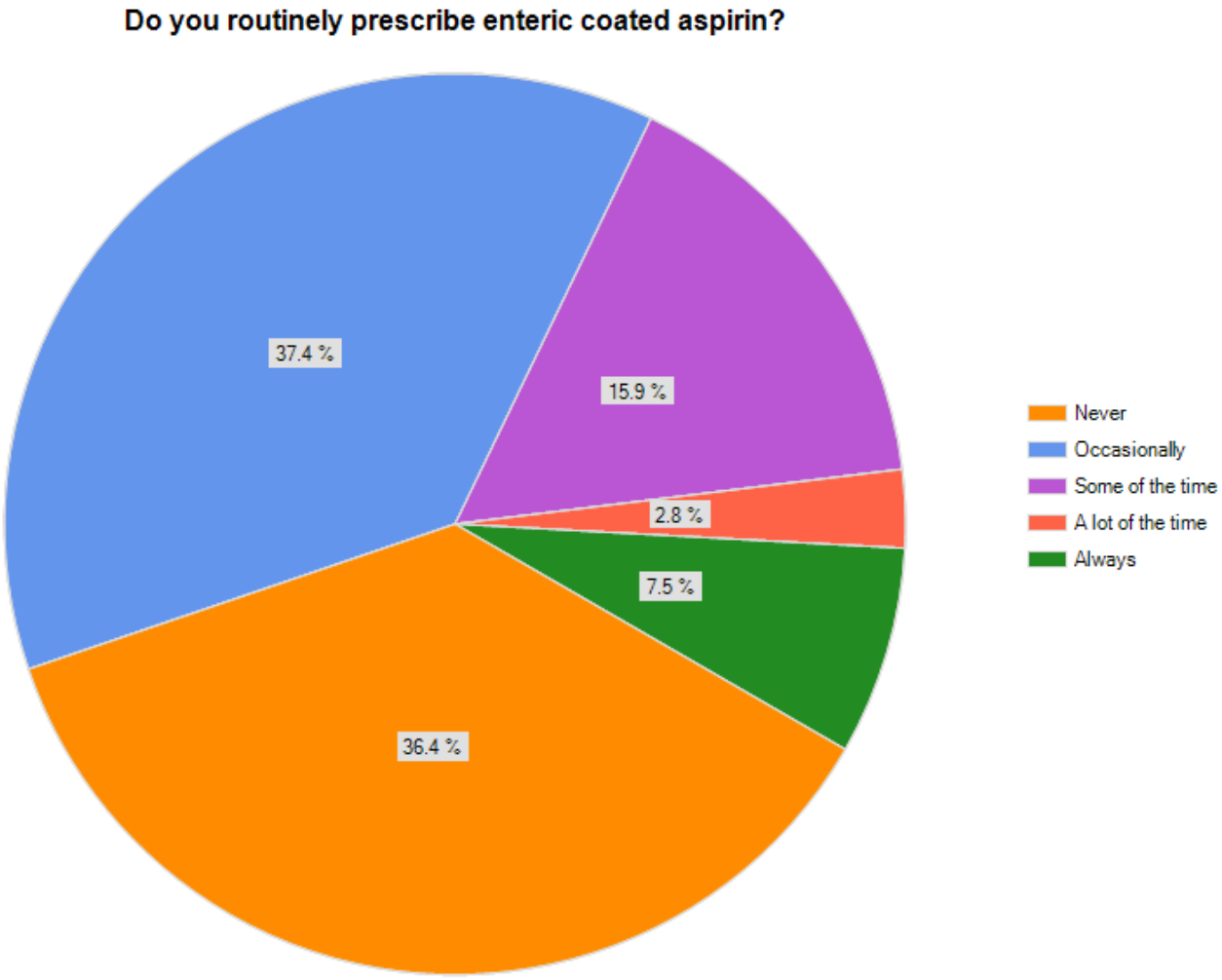


Figure-15: Use of enteric coated aspirin in clinical practice amongst participants

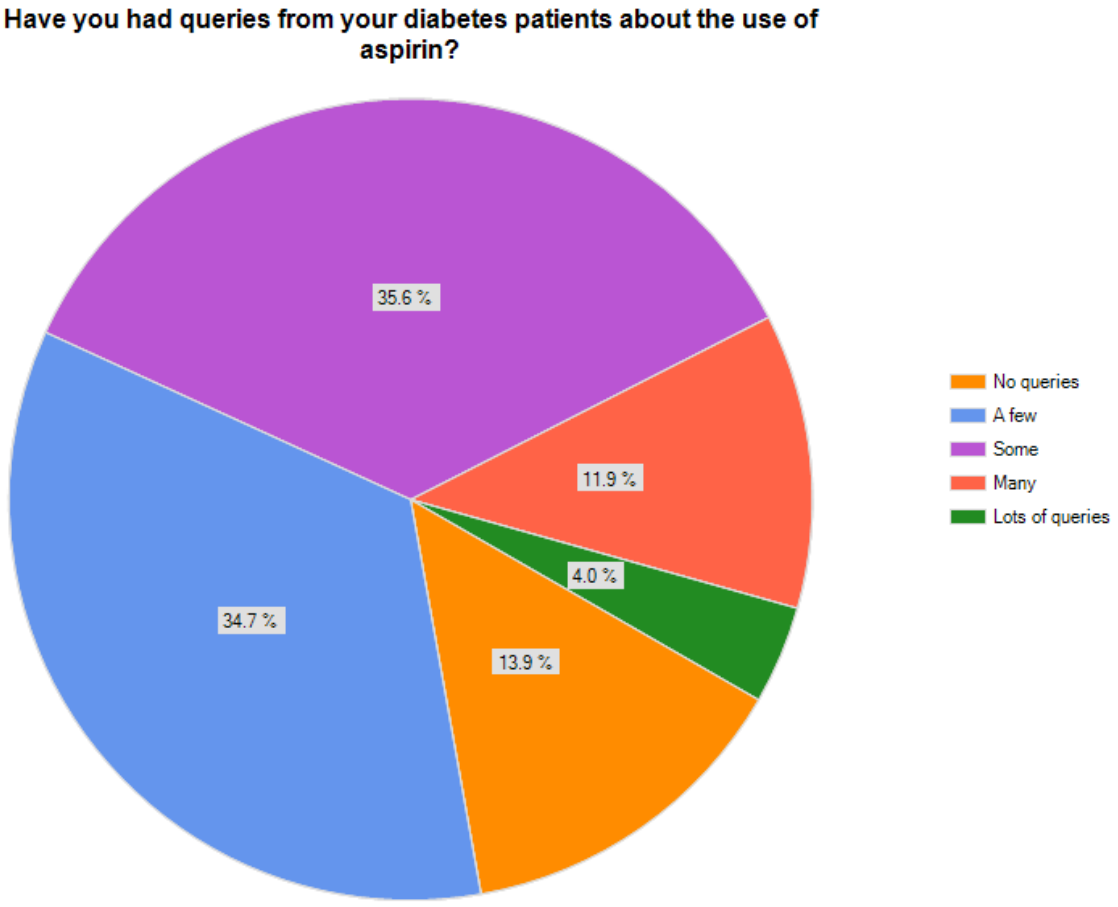


Figure-16: Queries about aspirin use from patients

There has been a lot of press regarding aspirin and this was borne out by the fact that 35.6% of respondents were approached at least some of the time regarding aspirin and 16% reported lots of queries from patients (see Figure 16). 26.7% of survey participants would definitely take aspirin themselves and 39.6% would consider it depending on their clinical circumstances (see Figure 17). There were no significant differences by groups when these answers were analysed.

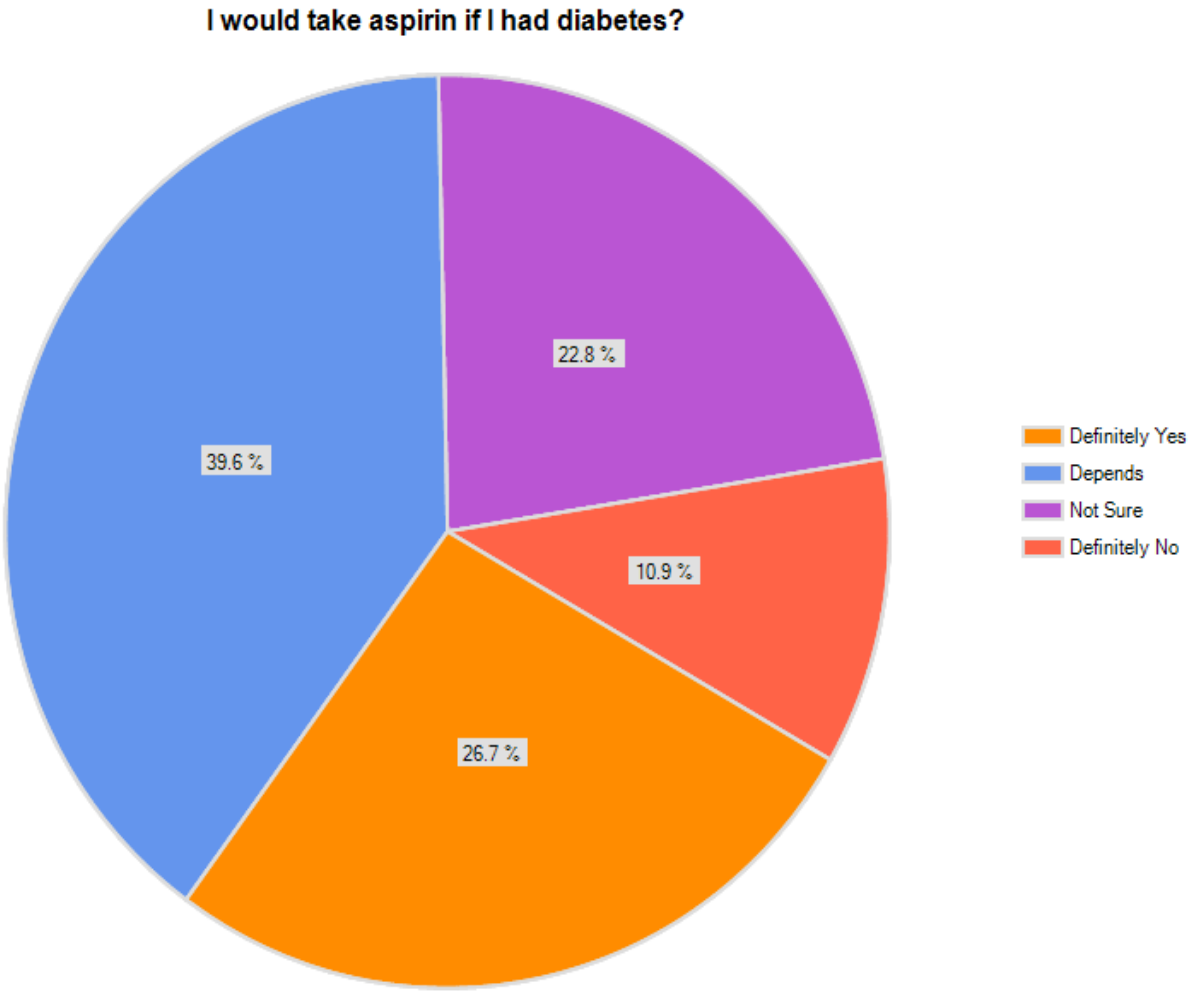


Figure-17: Views on personal use of aspirin by participants in survey

Section 2 – Metabolic Markers

a) Baseline Characteristics:

The baseline information for study participants are recorded in table-7 below. 21 subjects entered the study. Four of these dropped out due to personal reasons or change in medications necessitated by their co-morbidities which excluded them from the study. 17 subjects completed the study and all subsequent reporting is on this cohort. Of these 12 subjects were male and 5 were of female gender. The average age of study subjects were 59.4 ± 6.6 years, average weight was 90.35±20.51 Kg (Mean±1SD), average body mass index was 31.48±5.56 Kgm^{-2}, and average HbA1c was 7.81±1.18 %. Glycated haemoglobin was carried out at baseline and then as clinically indicated. Fructosamine was used a marker of short term (over 2 weeks) changes in glycaemic control. Further baseline characteristics are set out in Appendix-B.

Table-7: Demographics of participants including weight (Kg) at baseline, body mass index (KgM^{-2}), blood pressure (BP) & glycated haemoglobin (HbA1c, DCCT aligned)

Demographics	Age (Yrs)	Sex	Weight (Kg)	BMI (KgM^{-2})	BP (mmHg)	HbA1c (% DCCT)
DiASP-1	59	M	94.60	29.86	140	7.00
DiASP-2	65	M	140.60	42.92	146	6.90
DiASP-3	66	M	98.10	31.63	140	7.30
DiASP-4	57	M	91.90	34.17	139	6.40
DiASP-5	60	M	72.00	28.27	126	6.70
DiASP-6	60	F	60.90	24.55	137	6.70
DiASP-7	59	F	77.60	28.57	119	6.60
DiASP-8	58	F	78.40	31.73	115	7.20
DiASP-9	64	M	123.60	40.73	130	8.50
DiASP-10	53	M	101.80	26.66	126	8.00
DiASP-11	67	M	97.60	32.42	125	8.10
DiASP-12	60	M	121.80	40.84	139	9.80
DiASP-13	69	M	105.80	33.39	114	8.70
DiASP-14	53	F	86.80	30.75	120	8.80
DiASP-15	62	M	73.20	24.18	149	9.50
DiASP-16	63	M	84.00	26.81	150	7.80
DiASP-17	41	F	95.60	35.11	120	10.10

b) Results of baseline metabolic markers: (See also Table 28, P113)

All values are reported in Mean±SD unless data are non-parametric when Median (& interquartile range) is quoted.

BMI: There was no statistically significant difference in BMI parameters with placebo or various doses of aspirin (P=0.19), with individual results reported in table-8.

Blood pressure: At baseline the mean systolic blood pressure was 130.97±11.9 mmHg (Mean±SD) and mean diastolic blood pressure was 73.7±7.7 mmHg (Mean±SD) suggesting good blood pressure control (see tables 8-10). There was no statistically significant difference in the systolic or diastolic blood pressures on repeated measures ANOVA (i.e. p-value>0.05).

Table-8: Body Mass Index (KgM^{-2}; ideal range 18-25) of participants over the course of the study.

Body Mass Index (Kgm^{-2})	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	29.86	29.42	29.73	30.43	29.86	29.89	30.39	29.26
DiASP-2	37.97	37.70	36.60	36.93	40.63	40.08	42.92	42.25
DiASP-3	31.76	31.63	31.73	31.73	31.12	31.60	31.63	31.76
DiASP-4	34.17	33.87	33.69	33.83	34.35	34.06	33.76	33.72
DiASP-5	28.27	28.38	28.66	28.46	28.50	27.91	28.03	28.27
DiASP-6	24.55	24.55	24.59	24.47	24.59	24.75	24.79	26.69
DiASP-7	28.39	28.20	28.28	28.43	28.35	28.17	28.57	28.20
DiASP-8	31.73	31.97	31.28	31.69	31.73	31.89	31.16	32.45
DiASP-9	43.10	40.43	40.73	40.86	41.55	41.72	41.55	42.67
DiASP-10	26.66	27.16	27.16	27.37	27.19	27.11	26.77	26.71
DiASP-11	32.59	31.92	31.59	31.82	32.42	32.39	31.63	31.49
DiASP-12	40.90	40.90	40.84	40.90	41.37	41.68	40.97	41.11
DiASP-13	33.96	34.34	33.71	32.70	33.46	33.52	33.39	34.40
DiASP-14	30.47	29.94	30.58	30.58	30.65	30.75	30.75	30.54
DiASP-15	23.78	24.94	24.97	24.21	24.18	24.47	24.44	24.57
DiASP-16	35.11	35.08	35.56	35.74	35.26	35.11	35.63	35.11
DiASP-17	26.91	26.88	26.81	26.84	26.81	27.20	27.26	27.07

Table-9: Systolic blood pressure readings in mmHg (average of 2 readings) for subjects, pre and post intervention.

Systolic BP readings (mmHg)	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	119.00	135.00	129.00	127.00	140.00	106.00	130.00	124.00
DiASP-2	112.00	108.00	127.00	113.00	127.00	125.00	146.00	147.00
DiASP-3	143.00	140.00	162.00	151.00	140.00	138.00	140.00	140.00
DiASP-4	139.00	128.00	144.00	124.00	140.00	126.00	120.00	120.00
DiASP-5	126.00	124.00	123.00	110.00	124.00	130.00	127.00	116.00
DiASP-6	137.00	135.00	135.00	135.00	147.00	148.00	126.00	139.00
DiASP-7	110.00	115.00	111.00	114.00	113.00	109.00	119.00	116.00
DiASP-8	133.00	93.00	117.00	91.00	115.00	104.00	94.00	122.00
DiASP-9	126.00	111.00	138.00	140.00	123.00	131.00	123.00	131.00
DiASP-10	126.00	111.00	138.00	140.00	123.00	131.00	109.00	116.00
DiASP-11	124.00	124.00	134.00	143.00	125.00	136.00	139.00	139.00
DiASP-12	147.00	117.00	139.00	151.00	120.00	124.00	123.00	115.00
DiASP-13	125.00	130.00	129.00	127.00	118.00	124.00	114.00	120.00
DiASP-14	153.00	133.00	137.00	162.00	149.00	133.00	120.00	112.00
DiASP-15	153.00	133.00	137.00	162.00	149.00	133.00	123.00	112.00
DiASP-16	120.00	122.00	116.00	114.00	121.00	121.00	128.00	119.00
DiASP-17	134.00	117.00	150.00	186.00	141.00	149.00	141.00	168.00

Table-10: Diastolic blood pressure readings in mmHg (average of 2 readings) for subjects, pre and post intervention.

Diastolic BP readings (mmHg)	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	75.00	81.00	78.00	79.00	85.00	68.00	76.00	75.00
DiASP-2	68.00	54.00	68.00	61.00	67.00	65.00	76.00	76.00
DiASP-3	62.00	78.00	86.00	85.00	74.00	72.00	70.00	83.00
DiASP-4	69.00	72.00	74.00	64.00	69.00	68.00	58.00	58.00
DiASP-5	67.00	75.00	75.00	62.00	75.00	76.00	72.00	62.00
DiASP-6	81.00	76.00	78.00	78.00	78.00	80.00	63.00	71.00
DiASP-7	72.00	74.00	74.00	73.00	70.00	69.00	78.00	70.00
DiASP-8	80.00	57.00	73.00	54.00	67.00	64.00	64.00	69.00
DiASP-9	65.00	67.00	66.00	65.00	62.00	67.00	65.00	71.00
DiASP-10	70.00	66.00	77.00	82.00	71.00	79.00	65.00	70.00
DiASP-11	64.00	64.00	71.00	75.00	63.00	78.00	75.00	74.00
DiASP-12	88.00	63.00	84.00	89.00	70.00	78.00	82.00	77.00
DiASP-13	81.00	75.00	78.00	83.00	76.00	74.00	70.00	73.00
DiASP-14	68.00	60.00	63.00	65.00	85.00	62.00	66.00	61.00
DiASP-15	92.00	86.00	78.00	94.00	87.00	84.00	70.00	69.00
DiASP-16	80.00	78.00	69.00	72.00	80.00	77.00	75.00	78.00
DiASP-17	69.00	62.00	80.00	75.00	70.00	77.00	74.00	79.00

Lipid profile: (Tables 11 & 12 and Table 28, P113)

94% (16/17) of the study subjects were on lipid lowering therapy with the majority on statins (one solely treated with fibrate- see Appendix B). At baseline total cholesterol was 4.23±0.84 mmol/l (≤5mmol/l), and average HDL cholesterol was 1.31±0.83 mmol/l (≥1mmol/l). This also suggests fairly good control overall in terms of lipids (JBS target for total cholesterol< 4mmol/L). Following treatment with placebo and different doses of aspirin there was no statistically significant difference between any of the treatment groups on repeated measures ANOVA ($P>0.05$).

Fructosamine: (see Table 13 and Table 28, P113)

At baseline serum Fructosamine was 278.77±50.6 mmol/l (ideal range <230mmol/L) and following treatment with placebo and various doses of aspirin the values were parametrically distributed. On repeated measures ANOVA there was no statistically significant difference following any intervention from respective baseline readings ($p=0.39$).

Table-11: Total cholesterol readings (in mmol/l) for subjects, pre and post intervention

Total Cholesterol (mmol/L)	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	2.97	3.57	2.93	3.51	3.21	3.26	3.85	3.12
DiASP-2	3.41	3.10	2.85	2.75	3.06	3.35	3.66	3.12
DiASP-3	4.54	4.54	4.80	4.70	4.46	4.39	4.44	4.63
DiASP-4	4.40	4.29	4.25	3.64	3.99	3.90	4.05	3.81
DiASP-5	6.28	5.29	5.46	5.80	6.52	4.90	6.31	5.83
DiASP-6	1.20	3.52	4.21	3.73	4.66	4.54	3.65	4.18
DiASP-7	3.32	3.59	4.08	3.77	3.83	3.65	4.14	3.51
DiASP-8	4.64	3.99	4.23	4.21	4.04	4.24	4.23	4.32
DiASP-9	3.60	3.07	4.40	4.10	3.96	4.23	4.13	3.37
DiASP-10	4.25	4.30	4.23	3.64	4.48	4.14	3.91	4.02
DiASP-11	3.94	3.61	4.51	4.20	4.32	4.46	3.48	3.61
DiASP-12	4.67	4.84	4.97	4.74	5.10	4.59	5.28	4.72
DiASP-13	3.85	3.93	3.95	3.89	3.62	4.38	3.90	3.86
DiASP-14	3.18	3.73	3.69	3.56	3.68	3.44	4.11	4.02
DiASP-15	5.70	5.46	4.80	5.05	5.52	4.68	5.88	5.50
DiASP-16	3.30	3.69	3.13	3.58	3.59	3.03	3.18	3.26
DiASP-17	4.98	4.93	6.33	5.27	4.67	5.90	4.83	4.71

Table-12: HDL cholesterol readings (in mmol/l) for subjects, pre and post intervention

HDL-C levels (mmol/L)	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	1.09	1.04	1.08	0.99	1.15	1.04	0.95	0.96
DiASP-2	2.40	1.80	1.59	1.29	1.99	2.30	0.79	0.75
DiASP-3	1.08	0.96	0.98	1.05	0.92	1.00	1.00	0.97
DiASP-4	1.08	0.99	0.93	0.90	0.89	0.87	0.93	0.89
DiASP-5	4.30	1.02	0.81	0.84	0.97	0.99	0.93	0.97
DiASP-6	0.92	0.82	0.79	0.83	0.83	0.78	0.86	0.84
DiASP-7	1.17	1.20	1.16	1.14	1.29	1.11	1.13	1.15
DiASP-8	1.87	1.33	1.71	1.34	1.39	1.41	1.33	1.41
DiASP-9	0.98	0.72	0.82	0.88	0.92	0.96	0.85	0.91
DiASP-10	1.05	1.07	1.17	1.00	1.14	1.14	1.04	1.03
DiASP-11	1.34	1.22	1.27	1.30	1.28	1.33	1.20	1.04
DiASP-12	1.02	0.98	1.01	0.99	0.97	0.95	1.02	0.99
DiASP-13	1.03	1.09	0.99	1.10	0.94	1.03	1.01	1.13
DiASP-14	1.42	1.35	1.33	1.25	0.68	1.22	0.66	1.31
DiASP-15	1.55	1.55	1.42	1.55	2.07	1.31	1.51	1.48
DiASP-16	1.02	1.06	1.44	0.99	1.01	0.96	0.94	0.91
DiASP-17	1.11	1.08	1.23	1.19	0.88	0.98	1.17	1.20

Table-13: Fructosamine levels (mmol/L) pre- and post- intervention in study subjects.

Fructosamine (mmol/L)	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	228.00	257.00	247.00	241.00	297.00	247.00	275.00	234.00
DiASP-2	212.00	206.00	213.00	200.00	205.00	199.00	247.00	208.00
DiASP-3	325.00	304.00	304.00	321.00	304.00	309.00	301.00	282.00
DiASP-4	243.00	237.00	262.00	234.00	233.00	238.00	245.00	238.00
DiASP-5	247.00	224.00	259.00	225.00	239.00	248.00	255.00	248.00
DiASP-6	273.00	268.00	277.00	307.00	285.00	274.00	254.00	272.00
DiASP-7	205.00	248.00	207.00	211.00	201.00	205.00	242.00	248.00
DiASP-8	291.00	275.00	312.00	290.00	265.00	281.00	296.00	282.00
DiASP-9	267.00	369.00	325.00	325.00	300.00	296.00	292.00	256.00
DiASP-10	278.00	281.00	247.00	272.00	261.00	272.00	240.00	261.00
DiASP-11	323.00	336.00	325.00	280.00	363.00	430.00	282.00	291.00
DiASP-12	280.00	309.00	326.00	319.00	308.00	293.00	329.00	311.00
DiASP-13	295.00	295.00	281.00	322.00	272.00	282.00	295.00	268.00
DiASP-14	395.00	331.00	343.00	326.00	353.00	404.00	364.00	334.00
DiASP-15	335.00	297.00	345.00	392.00	280.00	283.00	394.00	382.00
DiASP-16	264.00	307.00	303.00	320.00	292.00	294.00	322.00	292.00
DiASP-17	349.00	331.00	309.00	299.00	386.00	409.00	312.00	289.00

c) Markers of insulin sensitivity/resistance: (See also Table 28, P113)

Fasting insulin was measured by radioimmunoassay and the average of 3 samples 5 mins apart was taken as the insulin value for calculations and reporting (see table-14). Paired glucose samples were obtained at the same time points and the average of these values (table-15) was used for input into the HOMA 2nd generation calculator to obtain insulin sensitivity index (%S, see table-16). The calculator also gave a measure of beta cell mass (%B) the inverse of which is the insulin resistance measure (IR, see table-17).

The values were parametrically distributed both for %S and IR and there was a trend towards improvement with aspirin 75mg and aspirin 300mg but on repeated measures ANOVA there was no statistically significant difference for any of the doses of aspirin or placebo. The results are graphically outlined in figures 18 & 19.

Table-14: Serum insulin values (average of 2 readings) used for calculation of insulin sensitivity (%S) and beta cell function (%B)

Serum Insulin (mU/L)	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	5.95	5.40	2.40	0.80	7.80	10.50	8.00	4.73
DiASP-2	8.80	9.83	11.60	11.97	15.33	11.63	17.35	21.80
DiASP-3	12.40	8.87	9.60	8.67	4.53	4.43	8.85	5.03
DiASP-4	12.83	11.13	15.43	17.57	17.70	15.10	11.10	11.77
DiASP-5	4.10	4.67	10.67	17.07	6.73	6.93	9.67	3.90
DiASP-6	7.07	8.65	9.40	8.10	15.93	8.10	13.53	17.90
DiASP-7	9.67	7.60	10.83	11.67	4.30	3.07	6.85	3.50
DiASP-8	4.20	4.83	2.47	3.63	4.27	2.40	3.57	8.80
DiASP-9	11.53	14.03	6.77	4.60	4.77	9.63	11.13	16.83
DiASP-10	6.10	6.30	9.90	7.20	12.13	12.33	3.93	6.60
DiASP-11	8.07	3.27	9.97	11.03	6.30	10.25	11.57	14.27
DiASP-12	19.77	33.97	18.23	30.33	15.87	23.83	28.50	33.90
DiASP-13	8.93	11.97	23.83	12.03	19.57	12.53	10.73	14.70
DiASP-14	4.77	2.93	2.43	1.80	4.27	2.40	5.43	3.00
DiASP-15	3.87	5.80	6.93	4.63	7.97	7.23	6.63	7.77
DiASP-16	6.57	7.83	4.03	8.37	5.87	9.47	6.23	7.73
DiASP-17	31.10	27.70	27.10	30.30	30.43	37.00	43.80	31.30

Table-15: Measures of plasma glucose (average of 3 readings) used for calculation of insulin resistance via the HOMA method. Ideal glucose range (4-7 mmol/l)

Plasma Glucose (mmol/L)	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	8.55	7.90	7.27	8.63	7.33	9.05	9.83	8.57
DiASP-2	5.13	5.30	5.47	5.57	6.03	6.00	7.85	7.03
DiASP-3	10.00	7.37	9.30	11.17	11.40	11.53	6.45	7.73
DiASP-4	7.30	7.43	7.47	7.07	6.97	6.70	6.80	6.43
DiASP-5	7.70	6.93	6.53	6.40	6.67	6.70	6.73	6.27
DiASP-6	7.07	7.40	7.00	6.70	7.30	7.37	7.10	10.33
DiASP-7	5.77	6.83	6.03	8.50	7.67	7.60	8.50	9.03
DiASP-8	10.33	9.13	9.83	10.33	7.63	8.30	9.73	5.13
DiASP-9	8.07	13.80	7.63	9.97	8.43	8.13	8.83	9.03
DiASP-10	10.43	10.50	9.10	9.23	9.33	10.47	9.63	8.77
DiASP-11	7.27	8.50	7.67	7.90	10.90	10.75	7.30	7.23
DiASP-12	12.20	12.13	11.40	13.30	13.47	13.43	15.50	12.40
DiASP-13	5.90	5.30	10.67	8.40	7.20	6.67	6.73	8.33
DiASP-14	7.17	6.40	8.73	7.30	7.63	8.30	9.10	6.47
DiASP-15	7.43	7.27	9.40	8.07	7.77	7.27	9.13	8.90
DiASP-16	7.97	8.33	9.13	8.97	8.10	8.87	8.07	8.10
DiASP-17	8.17	6.87	8.70	9.17	9.17	12.60	8.27	10.63

Table-16: Measures of insulin sensitivity pre and post interventions calculated using the HOMA 2nd generation calculator from the Oxford Diabetes Trials unit.

Insulin Sensitivity %S	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	67.70	118.70	94.80	107.00	76.30	186.00	76.30	53.40
DiASP-2	34.40	28.70	56.80	54.80	75.90	67.40	42.00	55.00
DiASP-3	70.20	115.50	44.00	67.20	57.70	59.80	46.00	43.50
DiASP-4	55.30	55.30	47.00	53.70	39.10	35.10	35.00	41.30
DiASP-5	141.50	128.60	90.80	88.10	63.50	158.70	58.30	37.20
DiASP-6	85.00	68.70	64.50	75.60	38.20	73.40	45.10	30.60
DiASP-7	82.70	157.30	135.20	189.50	66.70	80.00	58.90	49.20
DiASP-8	136.40	188.30	101.60	203.80	185.30	196.10	124.40	209.30
DiASP-9	86.60	116.10	118.70	60.10	50.80	33.90	50.60	33.20
DiASP-10	86.60	83.60	137.20	84.90	114.20	99.00	56.40	76.70
DiASP-11	82.50	51.50	74.00	171.80	52.10	42.60	59.20	53.10
DiASP-12	29.00	16.50	26.00	15.80	30.10	104.50	15.00	15.60
DiASP-13	57.40	39.60	94.30	94.00	31.50	49.50	23.10	47.90
DiASP-14	150.90	75.90	178.10	144.80	112.10	109.10	125.50	113.90
DiASP-15	73.40	82.40	151.70	102.40	79.10	123.60	83.40	72.00
DiASP-16	21.90	19.40	14.30	17.90	19.60	23.00	19.30	14.30
DiASP-17	87.90	73.00	97.80	59.40	136.20	66.80	92.30	74.60

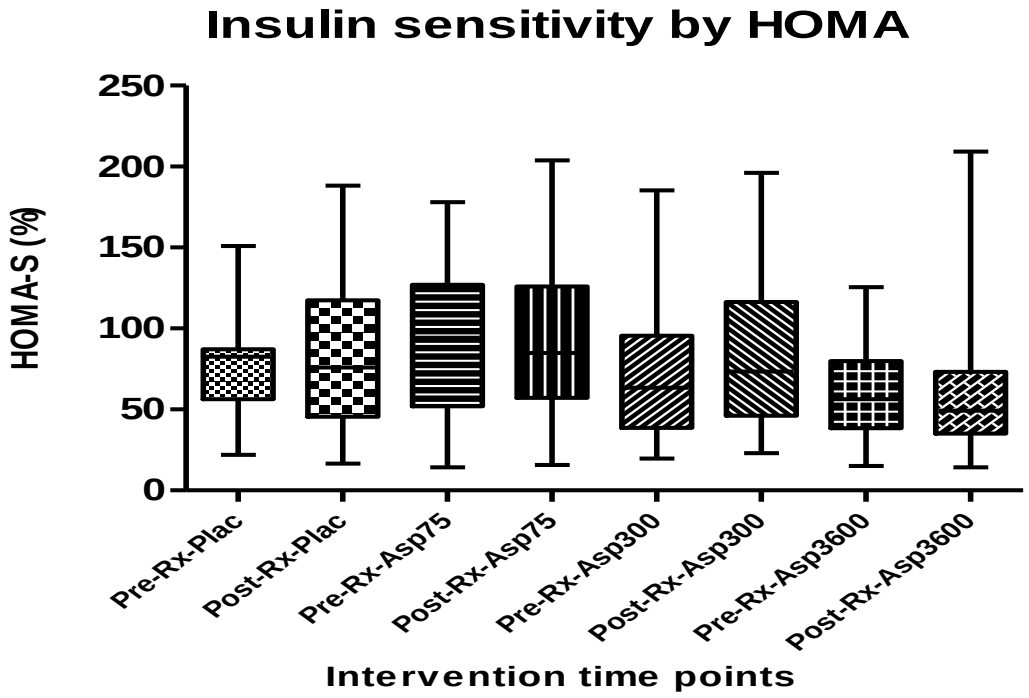


Figure-18: Insulin sensitivity results pre and post placebo and various doses of aspirin. Improvement with aspirin 75mg and aspirin 300mg doses but overall non-significant.

Table-17: Insulin resistance measures calculated as an inverse function of beta cell function (%B) using the 2nd generation HOMA calculator

Insulin Resistance (%B)	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	1.50	0.80	1.10	0.90	1.30	0.50	1.30	1.90
DiASP-2	2.90	3.50	1.80	1.80	1.30	1.50	2.40	1.80
DiASP-3	1.40	0.90	2.30	1.50	1.70	1.70	2.20	2.30
DiASP-4	1.80	1.80	2.10	1.90	2.60	2.80	2.90	2.40
DiASP-5	0.70	0.80	1.10	1.10	1.60	0.60	1.70	2.70
DiASP-6	1.20	1.50	1.60	1.30	2.60	1.40	2.20	3.30
DiASP-7	1.20	0.60	0.70	0.50	1.50	1.30	1.70	2.00
DiASP-8	0.70	0.50	1.00	0.50	0.50	0.50	0.80	0.50
DiASP-9	1.20	0.90	0.80	1.70	2.00	2.90	2.00	3.00
DiASP-10	1.20	1.20	0.70	1.20	0.90	1.00	1.80	1.30
DiASP-11	1.20	1.90	1.40	0.60	1.90	2.30	1.70	1.90
DiASP-12	3.40	6.10	3.80	6.30	3.30	1.00	6.70	6.40
DiASP-13	1.70	2.50	1.10	1.10	3.20	2.00	4.30	2.10
DiASP-14	0.70	1.30	0.60	0.70	0.90	0.90	0.80	0.90
DiASP-15	1.40	1.20	0.70	1.00	1.30	0.80	1.20	1.40
DiASP-16	4.60	5.20	7.00	5.60	5.10	4.30	5.20	7.00
DiASP-17	1.10	1.40	1.00	1.70	0.70	1.50	1.10	1.30

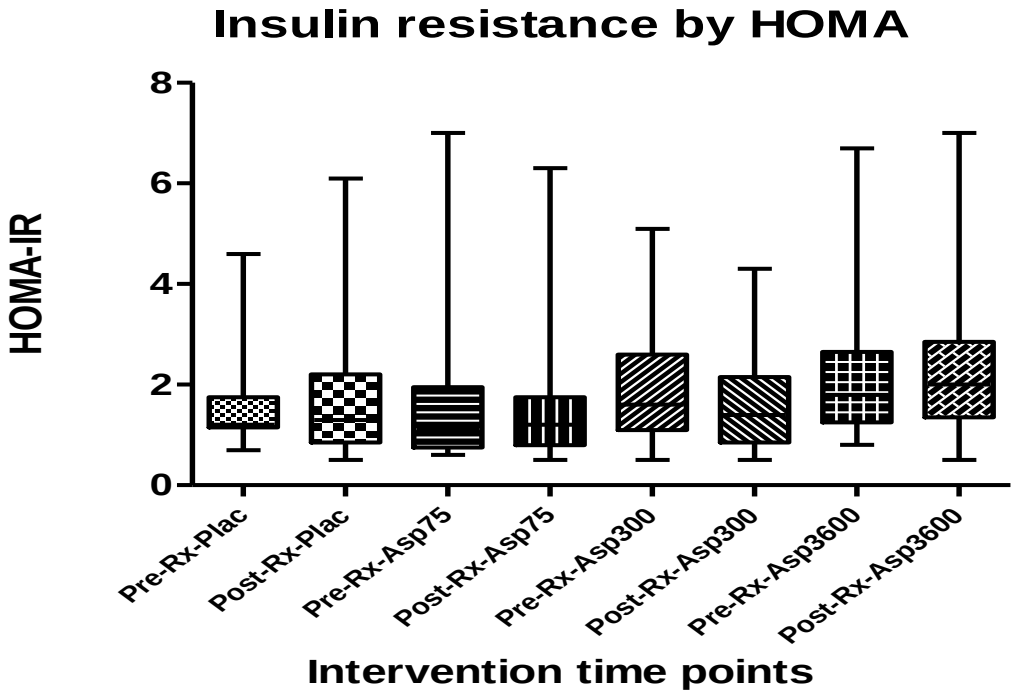


Figure-19: Insulin resistance results pre and post placebo and various doses of aspirin. Improvement with aspirin 75mg and aspirin 300mg doses but overall non-significant.

Section 4 – Markers of Antioxidant defence/function

Measures of anti-oxidant defence/function included plasma total anti-oxidant assay (TAOS assay using the ABTS+ method and the FRAP or Ferric reducing ability of plasma assay), and whole blood glutathione and ratio of total glutathione to reduced glutathione (GSH/GSSG).

ABTS+ assay method of total antioxidant capacity: The mean TAOS value reported as ascorbate equivalent anti-oxidant concentration (AEAC) represents antioxidant capacity of the plasma under scrutiny. At baseline TAOS concentration was 59.3±9.7 (ascorbate equivalent antioxidant concentration or AEAC (μM), Mean±1SD). There was a trend towards improvement in the overall TAOS assay (see table-18 and Table 28, p114) with repeated measures ANOVA, but this did not reach significance ($p>0.05$), see graphical representation of results in figure-20.

Table-18: Plasma TAOS values (in Ascorbate Equivalent Concentrations micromolar [AEAC μM]) from the total antioxidant assay pre and post intervention.

TAOS assay (AEAC μM)	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	66.00	57.77	62.86	59.95	50.48	64.68	51.87	58.42
DiASP-2	65.92	66.80	66.80	70.22	64.90	58.71	70.00	68.84
DiASP-3	63.74	63.08	71.31	66.72	64.17	68.91	61.41	62.35
DiASP-4	66.94	66.21	60.46	60.68	64.25	77.90	62.43	60.53
DiASP-5	45.31	66.43	61.41	63.08	56.53	63.37	50.26	59.66
DiASP-6	46.91	51.50	18.22	58.64	56.24	52.23	47.13	50.92
DiASP-7	48.73	61.63	56.24	65.41	57.98	63.59	38.76	47.35
DiASP-8	68.62	59.88	65.92	67.89	65.05	67.82	65.05	66.94
DiASP-9	67.53	68.91	66.36	67.53	67.31	65.19	64.90	68.47
DiASP-10	65.12	67.67	65.70	66.58	62.79	67.53	66.07	65.63
DiASP-11	63.16	64.54	66.72	62.72	67.82	62.72	64.54	65.92
DiASP-12	60.90	52.89	48.44	59.00	52.16	63.81	54.56	48.44
DiASP-13	66.29	66.14	60.17	63.88	62.28	80.50	66.00	66.58
DiASP-14	69.27	67.82	67.60	68.54	69.86	68.84	66.87	66.58
DiASP-15	59.22	68.76	65.56	64.03	67.16	66.94	62.06	66.87
DiASP-16	69.49	70.22	58.71	70.00	70.00	70.51	67.60	69.13
DiASP-17	68.47	67.67	67.96	67.31	65.63	58.42	67.38	65.05

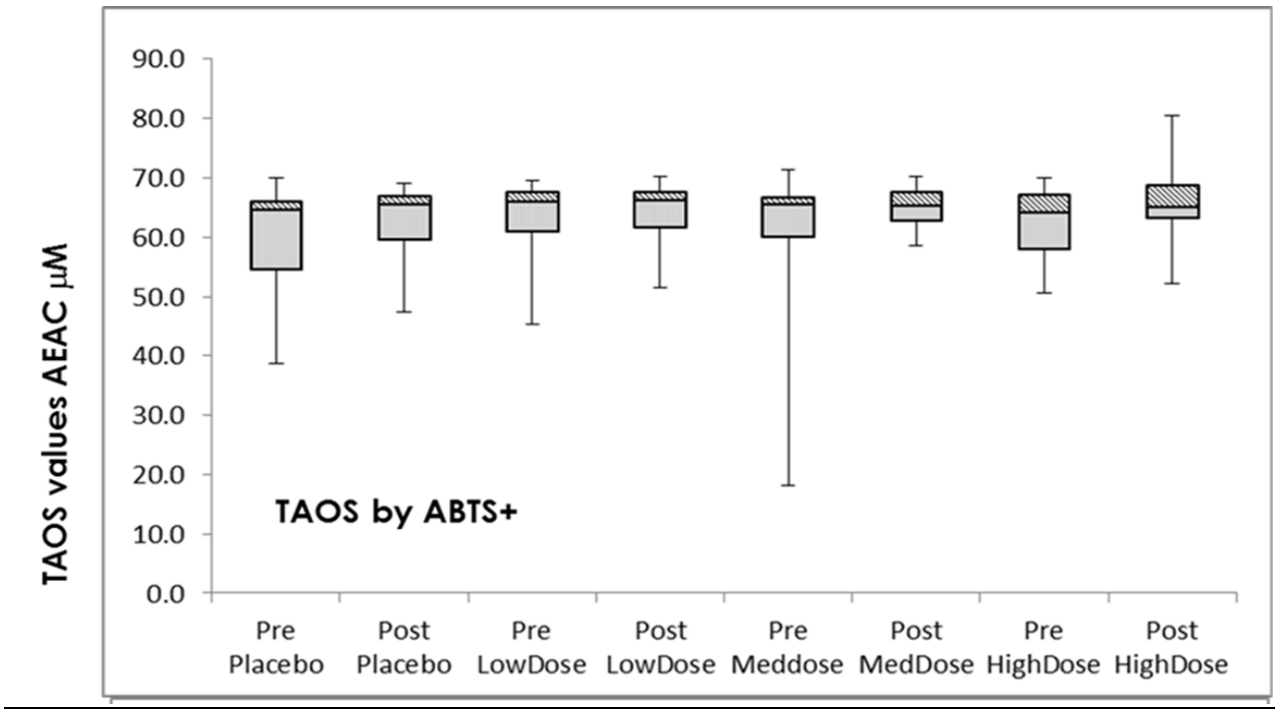


Figure-20: Graphical representation using BOX plots of TAOS values following placebo and different doses of aspirin

Table-19: Values derived from the Ferric reducing ability of plasma (FRAP) assay reported in μM Fe II.

FRAP assay (μM Fe II)	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	0.91	0.97	0.94	0.79	1.42	0.88	1.05	0.96
DiASP-2	0.77	0.82	0.81	0.75	0.96	0.84	0.95	0.77
DiASP-3	0.69	0.62	0.77	0.71	0.78	0.67	0.78	0.70
DiASP-4	0.70	0.75	0.65	0.63	0.67	0.79	1.05	0.67
DiASP-5	1.12	1.19	0.85	0.80	1.12	1.01	0.87	0.82
DiASP-6	1.09	0.82	0.79	0.77	1.28	0.72	0.72	0.94
DiASP-7	0.48	0.54	0.62	0.83	0.52	0.62	1.00	0.73
DiASP-8	0.64	0.69	0.61	0.81	0.48	0.63	0.59	0.63
DiASP-9	0.64	0.96	0.87	0.74	0.71	0.72	0.60	0.67
DiASP-10	0.85	0.67	0.85	0.77	0.85	0.57	0.98	0.88
DiASP-11	0.92	0.98	0.69	0.55	0.97	0.94	0.69	0.57
DiASP-12	0.83	0.96	0.78	0.77	0.92	0.89	0.91	0.89
DiASP-13	0.48	0.56	0.46	0.54	0.48	0.39	0.62	0.37
DiASP-14	0.55	0.54	0.42	0.46	0.54	0.57	0.42	0.40
DiASP-15	0.75	0.60	0.60	0.55	0.61	0.82	0.54	0.53
DiASP-16	0.90	0.81	0.82	0.89	0.97	0.79	1.12	1.01
DiASP-17	0.62	0.74	0.64	0.00	0.75	0.69	0.73	0.66

Ferric reducing ability of plasma (FRAP) assay: The average value for this assay at baseline as 0.80±0.25 μM Fe II (Mean±1SD). The differences on treatment with placebo or different doses of aspirin were not statistically significant ($p>0.05$).

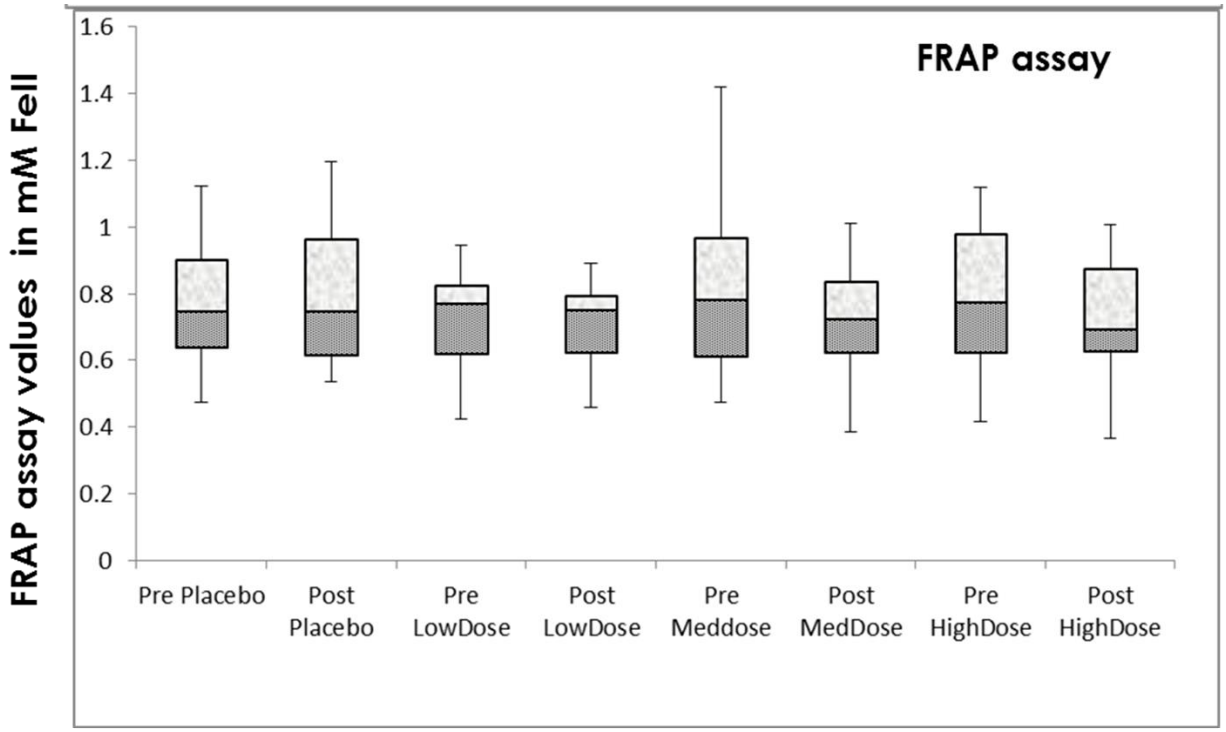


Figure-21: FRAP assay results graphically represented.

Glutathione assay: Whole blood equivalents of glutathione concentration were worked out by derivatisation and the mean oxidized glutathione concentration (GSH+GSSG) at baseline was 302.2±183.3 (µM, Mean±1SD). Reduced glutathione concentration (GSH) at baseline averaged at 241.08±176.04 (µM, Mean±1SD). The average of the ratio (GSH:GSSG) at baseline was 7.54±5.1 (Mean±1SD). None of these parameters were significantly different when pre-treatment and post-treatment values were analysed with either placebo or different doses of aspirin.

Table-20: Total glutathione values from whole blood pre and post interventions.

Total Glutathione (μM)	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	264.37	117.87	164.76	206.23	301.96	240.50	159.52	385.51
DiASP-2	234.95	77.44	249.55	124.73	217.32	120.04	176.94	180.95
DiASP-3	92.64	62.66	167.66	129.30	413.16	191.14	214.70	247.29
DiASP-4	462.47	81.87	55.83	479.00	32.18	382.02	151.30	183.63
DiASP-5	213.70	507.65	99.96	550.60	359.03	304.98	302.96	348.63
DiASP-6	133.08	245.66	201.90	290.72	258.34	365.28	334.33	381.10
DiASP-7	113.85	230.87	291.58	536.19	269.90	401.53	137.84	126.81
DiASP-8	397.64	190.11	186.26	208.66	426.76	334.74	462.16	113.41
DiASP-9	245.23	51.45	323.83	358.26	328.55	270.77	266.56	286.81
DiASP-10	141.20	249.37	565.78	245.16	50.52	140.63	389.88	39.92
DiASP-11	303.10	111.75	148.47	571.41	605.48	118.75	590.69	23.90
DiASP-12	268.14	175.76	517.05	569.09	185.17	535.02	283.25	140.16
DiASP-13	203.30	684.93	424.39	255.06	210.22	168.17	329.83	198.90
DiASP-14	271.61	373.63	432.87	172.57	160.64	177.27	424.42	265.92
DiASP-15	831.90	200.17	130.72	842.12	456.46	570.54	345.63	524.74
DiASP-16	121.71	281.03	579.07	545.22	967.72	600.72	402.23	160.50
DiASP-17	341.65	150.13	286.21	219.13	269.48	218.54	42.74	64.15

Table-21: Reduced glutathione (GSH) values (μM) pre and post intervention with placebo and aspirin.

Reduced Glutathione (μM)	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	220.48	96.62	145.63	163.01	237.83	166.94	102.26	349.18
DiASP-2	220.97	57.72	225.56	110.08	158.49	93.19	154.04	133.86
DiASP-3	51.20	52.67	105.27	104.36	397.82	175.94	145.53	209.95
DiASP-4	454.15	62.15	30.73	468.85	26.89	346.20	133.71	157.32
DiASP-5	180.06	474.67	83.89	538.68	309.02	265.28	291.52	317.45
DiASP-6	104.32	199.86	145.64	240.98	231.42	352.04	296.35	362.25
DiASP-7	72.75	182.02	266.12	501.15	224.56	341.88	61.46	54.05
DiASP-8	356.44	178.58	158.02	181.88	390.22	306.15	388.56	82.80
DiASP-9	199.40	35.51	285.78	280.71	285.76	213.11	222.04	249.40
DiASP-10	98.93	211.36	557.91	207.42	14.84	118.25	305.75	39.30
DiASP-11	282.06	88.94	126.59	546.21	568.78	95.07	558.94	18.89
DiASP-12	249.11	159.90	499.76	545.43	175.88	514.16	256.31	120.89
DiASP-13	198.33	650.43	419.95	240.49	147.96	156.38	326.04	184.33
DiASP-14	225.37	339.50	414.93	157.05	131.86	136.30	395.99	191.10
DiASP-15	786.82	183.63	102.99	768.58	413.17	527.94	311.43	447.29
DiASP-16	92.90	254.85	548.98	533.31	938.71	592.54	376.61	133.12
DiASP-17	305.05	98.28	248.08	189.01	222.90	177.13	27.06	51.57

Table-22: Glutathione ratio (whole blood equivalents) calculated from the ratio to GSH/GSSG.

Glutathione ratio	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	5.02	4.55	7.61	3.77	3.71	2.27	1.79	9.61
DiASP-2	9.21	3.94	3.83	4.10	11.34	4.73	6.73	2.84
DiASP-3	1.24	5.27	1.69	4.18	25.95	11.58	2.10	5.62
DiASP-4	54.58	3.15	1.22	46.22	5.08	9.66	7.60	5.98
DiASP-5	5.35	14.39	5.22	45.19	6.18	6.68	25.48	10.18
DiASP-6	3.63	4.36	2.59	4.85	8.60	26.60	7.80	19.22
DiASP-7	1.77	3.73	10.45	14.30	4.95	5.73	0.80	0.74
DiASP-8	8.65	15.49	5.60	6.79	10.68	10.71	5.28	2.71
DiASP-9	4.35	2.23	7.51	3.62	6.68	3.70	4.99	6.67
DiASP-10	2.34	5.56	70.91	5.50	0.42	5.28	3.63	63.60
DiASP-11	13.40	3.90	5.79	21.68	15.50	4.02	17.61	3.77
DiASP-12	13.08	10.08	28.90	23.06	18.94	24.65	9.51	6.27
DiASP-13	39.88	18.85	94.43	16.50	2.38	13.27	86.04	12.66
DiASP-14	4.87	9.95	23.14	10.11	4.58	3.33	13.92	2.55
DiASP-15	17.46	11.10	3.71	10.45	9.54	12.39	9.11	5.78
DiASP-16	3.22	9.73	18.25	44.75	32.36	72.47	14.70	4.86
DiASP-17	8.33	1.90	6.50	6.27	4.79	4.28	1.73	4.10

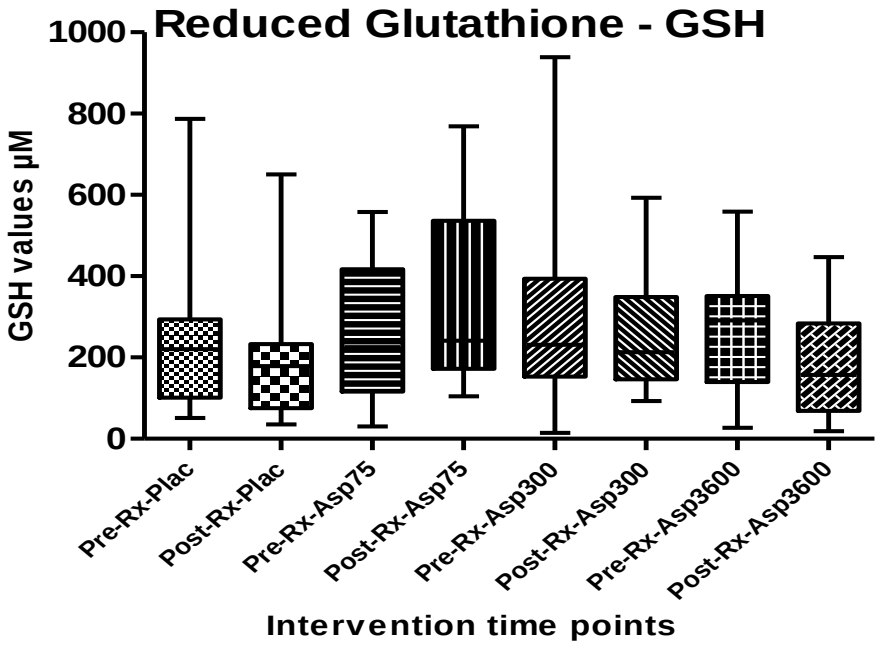


Figure-22: Graphical representation of whole blood reduced glutathione (GSH) in µM following placebo and various doses of aspirin

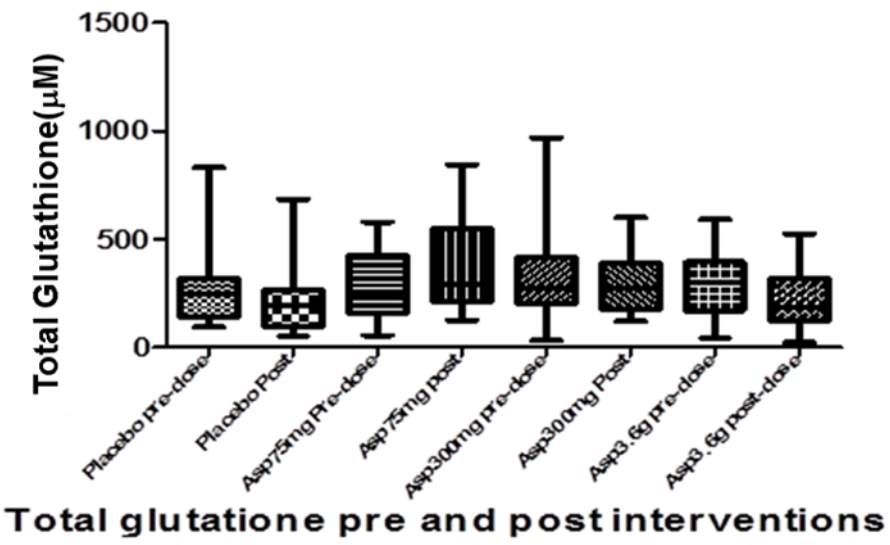


Fig 23: Graphical representation of whole blood total glutathione (GSH+GSSG) in µM following placebo and various doses of aspirin

Section 4 – Markers of Vascular Inflammation

These include highly sensitive C-reactive protein (Hs-CRP) and soluble vascular adhesion molecule (sVCAM-1).

HS-CRP results: (See also Table 28, P113)

The Hs-CRP assay was assayed in a sub-group of 15 patients as listed in table-23 below. This was due to technical issues with sample availability in the remaining subjects. The average HsCRP at baseline was 0.66 mg/L (0.41 to 2.06 mg/L, Median & IQR). Data was non-parametric on applying the Kolmogorov-Smirnov test of normality. Friedman's non-parametric repeated measures ANOVA showed that there was no statistically significant change in HsCRP values with any of the doses of aspirin or placebo (P=0.62).

Table-23: Results from the serum Hs-CRP assay pre and post interventions for 15 out of 17 subjects.

Hs-CRP assay (mg/L)	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	0.27	0.41	0.97	0.29	0.41	0.40	0.30	0.32
DiASP-2	0.22	0.15	0.66	0.37	0.32	0.31	0.94	2.32
DiASP-3	0.72	1.01	0.74	0.91	0.71	1.23	0.94	0.67
DiASP-5	0.46	2.09	0.38	0.53	1.42	0.33	0.73	0.50
DiASP-6	1.64	1.34	1.37	1.23	1.98	1.49	1.47	2.40
DiASP-7	2.65	1.67	2.03	2.80	2.05	4.12	2.68	2.63
DiASP-8	0.42	0.41	0.57	0.42	0.29	0.35	0.74	0.43
DiASP-9	0.64	0.82	2.70	0.70	0.65	0.82	0.45	14.40
DiASP-11	1.00	0.97	1.60	1.54	1.05	0.84	0.85	1.00
DiASP-12	4.88	6.14	5.22	4.77	8.31	4.62	4.37	6.56
DiASP-13	0.66	0.58	0.44	0.64	0.62	0.61	0.55	0.94
DiASP-14	0.39	0.42	0.33	0.41	0.65	0.49	0.45	0.23
DiASP-15	0.15	0.44	0.37	0.59	0.80	0.69	0.49	0.34
DiASP-16	2.49	1.97	2.08	4.09	1.82	2.12	2.47	3.87
DiASP-17	6.71	5.07	7.99	17.90	8.30	11.50	6.01	7.37

Human sVCAM-1 assay results: (See also Table 28, P113)

s-VCAM-1 was analysed from samples gathered from 15 of the 17 subjects (see Table-24) due to technical issues with sample availability in the remaining subjects. The results were non-parametrically distributed on application of the Kolmogorov-Smirnov test. The median s-VCAM-1 value at baseline was 487 ng/ml (IQR = 450.4 to 572.3) with normal range being 341-897 ng/ml. There was no statistically significant difference between the various doses of aspirin or placebo on application of the Friedman's test (P=0.813).

Table-24: sVCAM-1 assay results from 15 of the 17 subjects. The other 2 subjects were excluded due to insufficient samples available for analysis.

sVCAM-1 assay values (ng/ml)	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	515.18	494.65	533.04	499.11	521.43	633.93	522.33	558.93
DiASP-2	464.90	508.52	584.36	1050.81	472.28	452.15	436.04	470.94
DiASP-3	553.01	542.44	503.42	513.17	538.38	478.21	544.07	430.24
DiASP-4	465.20	364.39	347.32	376.59	394.47	422.11	374.96	405.04
DiASP-6	382.15	450.01	425.01	490.18	435.72	521.43	548.22	503.58
DiASP-8	392.42	393.76	384.36	401.81	376.31	355.50	397.11	508.52
DiASP-9	575.01	463.40	537.51	491.97	628.58	627.68	491.08	564.29
DiASP-10	237.50	451.79	297.33	405.36	457.15	415.18	491.97	458.93
DiASP-11	461.61	482.15	490.18	524.11	429.47	450.90	465.18	416.08
DiASP-12	569.60	520.60	541.41	551.48	497.11	456.18	546.11	501.14
DiASP-13	788.78	853.01	769.27	822.93	721.30	840.00	832.68	778.21
DiASP-14	439.19	483.09	543.25	675.77	518.05	570.08	583.09	460.33
DiASP-15	487.05	456.18	338.05	354.16	511.21	478.99	456.18	481.01
DiASP-16	668.93	646.11	558.86	703.16	616.58	583.02	607.18	579.67
DiASP-17	640.00	942.44	722.12	602.60	857.07	1142.44	679.03	793.66

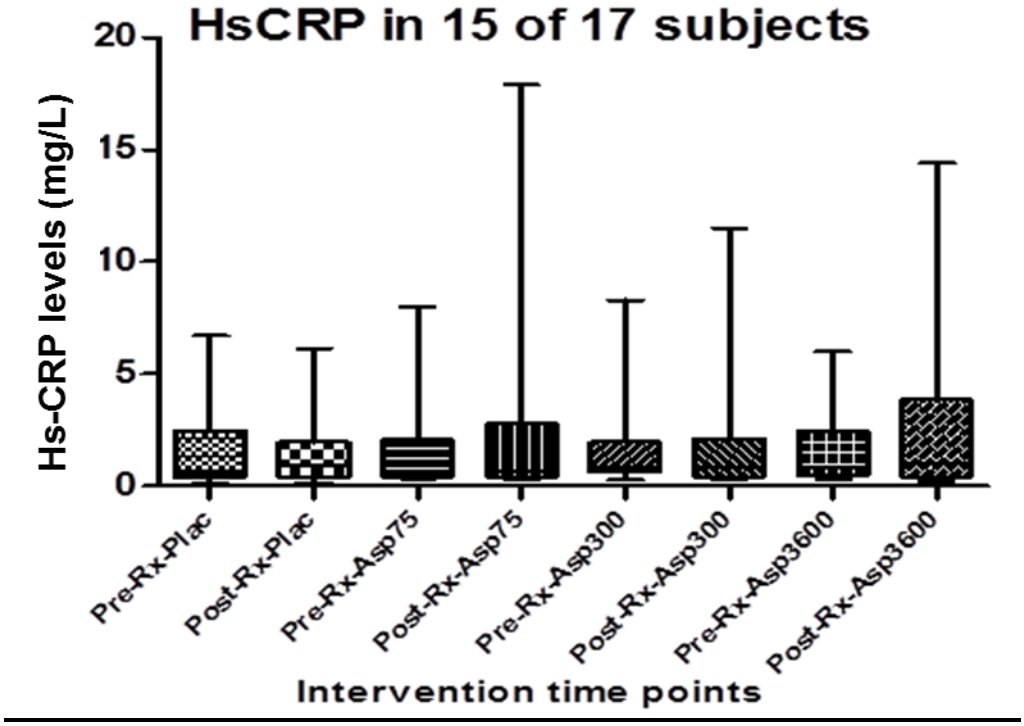


Figure-24: Serum HsCRP levels in mg/L following placebo and different doses of aspirin in a subset of 15 subjects.

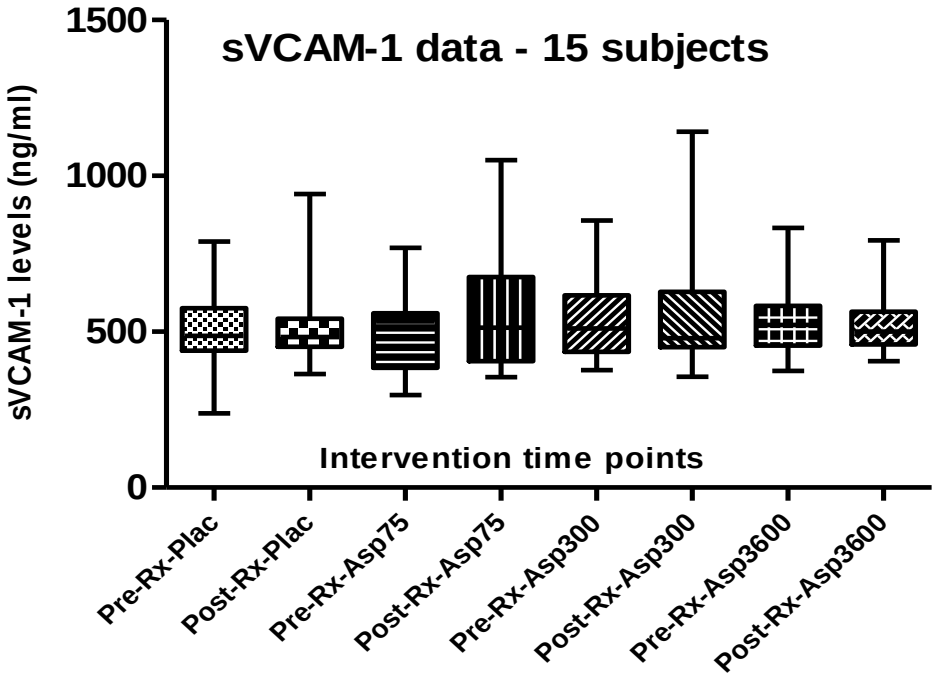


Figure-25: Plasma sVCAM-1 levels in ng/ml following placebo and different doses of aspirin in a subset of 15 subjects.

Section 5 – Endothelial Function via Photoplethysmography

The baseline readings from photoplethysmography were averaged over 3 readings for reporting purposes. Readings following GTN and Salbutamol were then taken and the average values subtracted from the baseline readings (RIΔ) (see table-25) to obtain the reflectance index for GTN (RI-GTN, see table-26 and table 28) and salbutamol (RI-Salb, see table-27 and table 28). The values did not pass the Kolmogorov-Smirnov test of normality and hence the results are reported in median (& interquartile range).

At baseline the reflectance index of the digital volume pulse for GTN (RI-GTNbase) was 7.17 (3.17-12.83)% and post GTN for Aspirin 75 was 7.83(1.83-15.5)%, Aspirin 300mg – 10.33 (3.67-16)%, Aspirin 3.6g – 4.67 (0.67-11.17)% (see Table 28, P114) and following a non-parametric ANOVA with repeated measures there was no statistically significant difference between groups – $P>0.05$.

At baseline for salbutamol the median reading was 18.5 (13-21.5)5 and with Aspirin 75mg was 18.00 (10.17-18.5) %, Aspirin 300mg – 17.0 (7.33-23) %, and Aspirin 3.6gm/day – 15.17 (11.83-20.67) %. There was no statistically significant between the groups, $P>0.05$.

Table-25: Baseline readings of the reflectance index (RI-Δ) in % using photoplethysmography.

RI Baseline	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	66.67	66.67	66.00	55.67	59.33	66.33	55.67	61.33
DiASP-2	71.00	85.67	75.00	77.67	79.33	72.00	61.67	68.33
DiASP-3	67.33	76.33	64.67	70.00	78.33	66.33	64.00	68.67
DiASP-4	67.67	72.33	64.00	75.00	80.33	73.50	73.50	70.67
DiASP-5	82.33	76.33	72.00	77.33	84.00	79.00	71.00	77.00
DiASP-6	74.00	67.33	65.00	74.33	67.67	78.33	69.67	81.00
DiASP-7	60.00	61.33	62.00	58.00	59.67	54.00	63.33	50.67
DiASP-8	60.00	60.67	69.33	61.00	58.33	66.33	63.00	70.67
DiASP-9	79.00	72.00	59.67	71.67	69.33	89.00	76.67	81.00
DiASP-10	74.00	67.00	66.33	79.00	74.33	69.00	71.67	71.00
DiASP-11	85.33	79.00	87.00	76.67	75.67	67.33	80.00	85.33
DiASP-12	76.33	77.00	75.33	74.67	76.67	74.67	72.33	68.33
DiASP-13	68.67	73.67	76.33	70.67	74.67	69.67	71.00	70.67
DiASP-14	33.33	34.00	42.33	34.67	27.00	47.33	29.00	52.00
DiASP-15	51.00	61.67	73.67	71.33	56.33	64.00	67.00	61.67
DiASP-16	70.00	73.00	66.67	59.33	72.00	77.67	80.33	80.33
DiASP-17	71.33	60.67	39.33	42.33	38.67	39.00	66.67	66.67

Table-26: Reflectance index (%) Post GTN administration pre- and post- placebo and various doses of aspirin.

RI difference Post GTN	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	15.50	2.67	17.83	6.83	10.17	18.33	12.67	7.17
DiASP-2	21.50	24.67	19.33	26.00	18.00	11.17	8.17	6.33
DiASP-3	3.17	2.50	4.33	20.33	31.83	2.33	27.00	11.67
DiASP-4	18.50	-2.33	-8.83	7.83	6.83	18.50	-6.50	-3.00
DiASP-5	12.50	13.50	15.50	15.00	9.33	6.83	6.00	4.33
DiASP-6	7.17	10.50	7.50	2.83	2.50	0.83	8.17	25.33
DiASP-7	4.00	0.00	3.67	-4.50	4.50	11.83	8.33	-5.33
DiASP-8	12.83	24.33	16.33	8.50	18.50	17.17	8.00	16.17
DiASP-9	2.33	19.00	11.67	10.00	3.50	16.00	-3.33	11.17
DiASP-10	3.00	6.50	18.33	-6.00	3.33	17.50	18.00	6.00
DiASP-11	11.83	25.00	10.17	13.33	5.00	10.33	33.50	4.67
DiASP-12	1.83	1.00	1.83	-4.17	-2.67	0.67	0.17	0.67
DiASP-13	7.67	10.17	3.17	3.17	10.83	3.67	6.00	-0.83
DiASP-14	-1.50	9.83	-3.17	4.00	9.33	-8.33	8.00	3.00
DiASP-15	6.00	15.17	-2.33	22.83	13.00	13.67	3.83	4.00
DiASP-16	18.67	11.67	-8.67	-0.67	33.33	8.17	-3.33	-7.00
DiASP-17	4.17	-8.17	9.83	25.33	5.50	8.67	6.50	18.00

Table-27: Reflectance index (%) Post salbutamol administration pre- and post- placebo and various doses of aspirin.

RI difference Post Salbutamol	Placebo		Aspirin 75mg/day		Aspirin 300mg/day		Aspirin 3.6grams/day	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
DiASP-1	26.67	25.17	29.00	19.17	22.33	22.33	14.67	13.83
DiASP-2	24.67	7.83	12.50	8.17	24.33	11.00	7.00	11.67
DiASP-3	4.50	8.17	13.67	8.50	11.83	9.33	18.83	11.83
DiASP-4	21.50	11.00	21.50	10.17	1.17	24.83	19.33	21.50
DiASP-5	8.50	16.33	16.50	17.00	24.50	18.50	14.83	13.83
DiASP-6	12.17	12.33	2.67	3.50	8.00	4.83	3.00	10.83
DiASP-7	23.33	17.17	18.50	18.00	17.67	23.00	25.00	13.83
DiASP-8	20.00	7.17	24.33	24.33	21.83	17.00	21.50	18.17
DiASP-9	6.17	20.67	8.33	19.50	13.17	10.00	15.50	21.50
DiASP-10	21.00	15.50	21.17	21.50	23.33	17.00	19.33	23.50
DiASP-11	17.50	13.17	10.83	18.50	13.17	1.33	14.50	22.33
DiASP-12	14.67	15.67	5.83	16.00	-2.17	3.83	15.33	15.17
DiASP-13	19.00	14.67	18.17	16.67	11.67	28.67	23.33	20.67
DiASP-14	13.00	16.00	12.50	18.33	10.83	23.50	17.33	5.17
DiASP-15	23.83	33.00	26.00	27.17	13.67	23.83	20.00	20.17
DiASP-16	13.67	14.00	22.17	22.17	9.83	4.83	12.83	11.67
DiASP-17	18.50	5.50	11.17	-7.67	17.33	7.33	15.50	16.67

Table-28: Summary of results for baseline, metabolic, vascular, and endothelial parameters at baseline and following different interventions.

Parameters studied		Baseline	Placebo	Aspirin Low dose	Aspirin Medium dose	Aspirin High dose
BMI (KgM^{-2})		31.4±5.4	31.3±5	31.6±5	31.9±5.4	32.1±5.6
Blood pressure (mmHg)	Systolic	131±11	122±12	135±24	128±13	127±15
	Diastolic	74±7	70±9	74±11	73±6	72±7
Total Cholesterol (< 4 mmol/L)		4.57±1.01	4.09±0.72	4.13±0.77	4.18±0.77	4.09±0.79
HDL Cholesterol (> 1.1 mmol/L in men and >1.2 mmol/L in women)		1.13±0.46	1.13±0.26	1.1±0.2	1.14±0.34	1.06±0.2
Fructosamine (205-285 µM/l)		282.9±50.6	286.76±43.54	287.29±50.88	292±66.01	276.24±40.81
HOMA-IR		1.41±1.04	1.89±1.61	1.73±1.65	1.59±1.02	2.48±1.74
HOMA-S %		70.27±45.2	83.48±48.43	93.58±55.13	88.74±52.34	60.05±45.41
TAOS (AEAC mM)		59.3±9.7	63.75±5.55	64.73±3.72	65.66±6.74	61.8±7.11
FRAP assay (mM Fe II)		0.86±0.23	0.78±0.19	0.71±0.12	0.74±0.16	0.72±0.19
GSH (µM)		241.08±176.07	195.69±162.91	339.84±201.5	269.32±157.36	182.52±125.96
GSH+GSSG (µM)		302.2±183.3	223.08±167.81	370.79±205.22	302.39±155.57	216.02±136.53
RI-GTN (%)		7.17 (3.17-12.83)	10.17 (2.15 – 15.17)	7.83 (1.83 – 15.5)	10.33 (3.67 – 16)	4.67 (0.67 – 11.17)
RI-SALB (%)		18.5 (13 – 21.5)	14.67 (11 – 16.33)	18 (10.17 – 18.5)	17 (7.33 – 23)	15.17 (11.83 – 20.67)
Hs-CRP (mg/dl), n=15		0.66 (0.41 – 2.06)	0.97 (0.43 – 1.52)	0.70 (0.48 – 2.17)	0.82 (0.44 – 1.81)	1.00 (0.47 – 3.25)
S-VCAM-1 (ng/ml), n=15		487.04 (450.4 - 542.3)	483.09 (453.98 - 531.52)	513.17 (447.77 - 639.19)	478.99 (451.52 – 605.35)	501.14 (459.63 – 561.61)

CHAPTER 4: DISCUSSION AND CONCLUSIONS

Section-1: Observational study, implications and limitations

The premise of the observational study was that a lack of clear guidance on aspirin use in diabetes was contributing to heterogeneous habits around aspirin prescription. The results of this study seem to confirm this with wide variation in aspirin prescription when weighed against conventional guidance. Differences were pronounced between primary and secondary care and may partly stem from the different populations that form the core of practice within these settings. Perhaps, this along with other factors such as a higher general perception of risk amongst secondary care practitioners of future vascular events, and guidelines recommending higher dose of aspirin following a cerebrovascular or cardiac event (300-600mg/day as an initial loading dose in acute coronary syndrome), may be responsible for the higher use of aspirin in this setting. Further analysis demonstrated that many of the key differences were seen between doctors and nurses and these differences could account for most of the variation between primary care and secondary care. The variation in approach to treatment may explain the differences between these 2 groups.

Aspirin as previously discussed has been universally accepted in secondary prevention (i.e. after an event has occurred such as a myocardial infarction, thrombotic stroke etc). There is very good evidence for its clinical value in this setting (69) and therefore one would expect near universal use in this situation except for those with contraindications or sensitivity to aspirin or where patient preferences would override any medical recommendations. However this survey found

that aspirin use was not recommended unanimously by study respondents (despite taking into account those with contraindications). This is borne out by previous work which reveals sub-optimal use of aspirin in various population settings.(78; 73) Similarly a more recent study assessing cardiovascular risk management in a high risk population with proliferative diabetic retinopathy found that aspirin was being used only in 35.3% of patients (as opposed to everyone in this group without contraindications to aspirin therapy).(181) This area gives pause for thought as there is clear deviation from recommendations (ADA position statement, 2004 (71)). It also raises questions regarding the clear understanding of differences between primary and secondary prevention and whether the tolerability and GI side effect issues with aspirin (and indeed other anti-platelets) are truly underreported. With the more recent studies reporting aspirin under-utilisation, one should also consider the degree of use of other medication such as clopidogrel or warfarin.

A majority of the respondents felt that type 1 and type 2 diabetes should be assessed as being similar in respect of aspirin use (see figure 11).However from the comments included with the responses to this question and reviewing the question retrospectively it is clear that given the earlier age of onset of type 1 diabetes and the later onset of macrovascular complications the approach to these 2 areas does differ in clinical practice. Overall there still seems to be some discrepancy between the recommendations in this area and the actual use in practice. Some of this may be explained by the use of other anti-platelet agents such as clopidogrel especially if there has been a further event while on aspirin. Concerns regarding aspirin and gastrointestinal bleeding may be behind some of the reluctance.

The question on aspirin use in patients with a history of peptic ulceration seems to state the contrary with only 6.9% of the respondents not willing to use anti-platelets and a further 22.8% wanting to use an alternative (see figure-9) although the risk of gastrointestinal bleeding is probably not that different with clopidogrel (182) and is particularly high in subjects who have a preceding history of GI bleeding (183) There is also some evidence that aspirin with concomitant PPI therapy may confer a lesser risk of GI bleeding compared to clopidogrel.(184) Further discussion regarding other anti-platelet agents is undertaken later in this chapter.

Our survey covered a small proportion of the UK wide diabetes fraternity. Local prescribing practices vary to some extent and this may have played a part in the heterogeneity of responses. Conversely this particular aspect could also highlight the differences of opinion even within a local network with regards to aspirin use in diabetes. Some of the survey respondents pointed out that certain questions could be answered differently depending on the particular patient one is faced with. While accepting this one would argue that the same is true of any guidance and that application may vary in practice. A previous survey of healthcare professionals' attitudes and practice in applying guidelines in secondary prevention of cardiovascular disease has demonstrated a positive attitude to guidelines.(185) However sub-optimal implementation of guidelines due to various barriers including lack of knowledge of guidelines and monetary considerations were also reported in the same study.(185) Cost considerations are definitely not a factor in aspirin prescription but knowledge of guidelines could play a part within any healthcare setting.

Some of the questions were not answered by all respondents and this could reflect a perceived ambiguity for the questions concerned. It is important to note that for the purposes of reporting we assumed a neutral response in these cases but the statistical analyses were performed with only data from those who answered to avoid reporting bias and the potential for a central tendency bias. Use of an online survey has both advantages and disadvantages. In particular we found that it was easy to distribute and administer the survey online and that the survey website avoided any tendency for "spatial" or "non-verbal" bias. The tendency for non-response / partial response is surprisingly not influenced by making the survey anonymous (186) and there is evidence to suggest that accuracy of response is improved with using 7 points as opposed to 5 points but has a tendency to make the survey of longer duration and increasing the tendency for poor completion rates. Previous work in this area has not clarified the utility of the neutral response element versus forcing a positive/negative response. If such a survey is undertaken again it is better to go for a forced positive/negative survey as prescribers or those able to influence prescribing should make a judgement either way and this may be a further flaw in the survey instrument used in this study.

Section 2 – Implications of Interventional findings

This study set out to investigate the differential effects of aspirin at various doses on pathophysiological markers in type 2 diabetes. None of the results reached statistical significance but there were distinct trends with the TAOS assay (but not FRAP assay) with numerical improvement in readings with aspirin 300mg and similarly with Insulin resistance (HOMA-IR)

and insulin sensitivity (%S) which did not reach statistical significance of <0.05%. The small population size in this study may be implicated.

Other studies have shown a small effect of low dose aspirin (75-100mgs) on total antioxidant capacity in serum in healthy human volunteers (88) and following exercise in subjects with peripheral vascular disease.(187) A cross sectional study in smokers however found that despite a reduction in urinary 11-dehydro-TxB_2 production and suppression of 8-epi-$PGF2_\alpha$ and TxB_2 in serum, urinary output of 8-epi-$PGF2_\alpha$, a marker of oxidative stress induced endpoint damage was not reduced with a single dose of aspirin of 325mgs.(188) A favourable change in insulin resistance (insulin clamp method) has been shown with very high dose aspirin 7.2 grams/day (51) but no previous studies have looked at effects of lower doses of aspirin on insulin resistance. Similarly endothelial function was alleviated by aspirin 1.2 grams when administered as pre-treatment in healthy individuals whose endothelial function was subjected to toxin mediated damage (93) but effects in subjects with diabetes at various doses have not been studied. Aspirin has been shown to lower HsCRP in those with high levels of HsCRP in the Physician's Health Study.(157) However in a recent study when used in combination with clopidogrel (CHARISMA) (189) there seemed to be no significant effect on HsCRP and paradoxically clopidogrel seemed to lower cardiovascular events compared to placebo in the group with the lowest levels of HsCRP. (189) While these findings demonstrate that aspirin is different from other anti-platelets they also provide conflicting information on the effectiveness of aspirin as an anti-inflammatory drug especially at lower doses. Furthermore these studies are all population based and may not be replicated at an individual level or in smaller scale studies over a shorter time period.

In this study aspirin did not show any effect on oxidative stress markers and it is widely acknowledged that an increased level of oxidative stress is known to occur in diabetes. Interestingly the baseline anti-oxidant capacity of serum measured by TAOS was actually higher in these study subjects compared with a previous study looking at middle aged men without diabetes or ischaemic heart disease (59 vs. 42 AEAC (µM)) (88) but lower compared to levels (92.6 AEAC (µM)) found in another study in a similar population with type 2 diabetes.(190) In contrast the levels of glutathione were lower in our subjects compared to a normal population assuming plasma glutathione reported in these studies were ~1% of the total (plasma GSH=7.87 mM/L as reported by Paroni et al., (147) 12.87 mM/L as reported by Pastore et al.,(191) and 1.02 mmol/L in whole blood as per Richie et al.,(192)). In general levels of glutathione are reduced in diabetes (193; 194) but in comparison to other studies in subjects with diabetes (190; 193) the levels found in this study were much lower. This could suggest higher levels of oxidative stress or lower anti-oxidant defence. Different studies have reported widely varying concentrations of glutathione levels (for e.g. from 86-2899 mcg/L as reported by Flagg et al.,(148)). Some of this variation is likely to represent differences in assay methodology and also as to whether plasma or whole blood glutathione is being measured. Other factors include age, sex, and dietary influences.(148; 192) Furthermore any haemolysis in samples would allow leakage of the GSH from erythrocytes which have high levels and thus lead to increased plasma levels. This could explicate the relatively modest changes in levels of oxidative stress in this study population for the intervention studied.

A Cycloxygenase independent mechanism contributing to aspirin-insensitive thromboxaneA_2 biosynthesis as previously shown, (89) could also explain the blunted anti-oxidant response with lower doses. Aspirin at high doses may prove detrimental by inhibiting PGI_2 production (195)

which could explain in part why high dose aspirin has not evoked any significant positive change upon oxidative stress markers in this study. Such mechanisms further raise the possibility of a potential dose-related effect. There is a suggestion that TxB_2 production with higher doses of aspirin especially in the setting of ACE-inhibitor treatment and heart failure can cause adverse effects. A majority of our subjects were on ACE-inhibitors/Angiotensin receptor blockers but none were known to have clinical evidence of heart failure.

There are various techniques, both direct and indirect, ex-vivo and in-vivo, of studying measures of oxidative stress and/or the body's defence against this insult (i.e. anti-oxidation). The TAOS and FRAP assays measuring anti-oxidant defence, and measurement of GSH/GSSG ratio (redox state) are a few of the well accepted methods used when dealing with human plasma/serum.(196) Differences between findings of the various assays selected for this study demonstrate intrinsic differences as these assays reflect different aspects of the oxidative stress/anti-oxidant system. (196) None of these markers should be studied in isolation and it is generally recommended that several assays be used simultaneously to measure oxidative status.

The changes in antioxidant defence (TAOS) seemed to parallel those in insulin resistance but the dose differences proved to be inconclusive. While the Homeostasis Model Assessment is well validated and reproducible, it reflects hepatic insulin resistance and does not fully account for peripheral insulin resistance.(141) Very high dose aspirin via inhibition of NF-κB is known to affect both peripheral and hepatic glucose resistance.(51) Subjects were off their anti-diabetic medication for the duration of this study conducted by Hundal et al. (51) while in our study all treatments were continued, which could be a significant factor influencing the changes in insulin resistance/sensitivity. Whether the inhibition of NF-κB is a dose-dependent effect and whether

different doses would affect the hepatic and peripheral insulin resistance differently could be the subject of further research in this area.

Section 3 – Confounding Factors and limitations of study

The study was conducted in a population with no cardiovascular disease at baseline but with high cardiovascular risk. While concentrating on a population with primary prevention at baseline such a population also has a lesser burden of metabolic risk factors and therefore it might be difficult to measure subtle changes that might have occurred over a short period of time such as in this study. Study subjects had respectable blood pressure control (SBP-130.8±11.5 mmHg; DBP-73.95±6.97 mmHg) with/without use of antihypertensive agents. In addition the average HbA1c-7.9±1.2% indicated good glycaemic control and lipid profile was also favourable (total cholesterol-4.57±1.01 mmol/l; HDL-C-1.13±0.46 mmol/l). A breakdown of the frequency of hypertension, use of anti-hypertensives (specifically renin-angiotensin system blocking agents) and lipid lowering therapy is set out in a table in Appendix-B. The high level of statin use (15/17, 88%) among study subjects also has relevance to vascular inflammation and endothelial function. It is well known that these factors have a direct impact upon the development of atherosclerosis and the health of the endothelium. (197)

An additional step in the dosing scheme (i.e.500-900 mgs) in order to probe a linear relationship to dose (especially in the 100-1000mgs/day range) was not feasible due to technical limitations. The anti-platelet effects of aspirin are seen at doses as low as 37.5mgs with doses above 75mgs showing no significant difference in anti-platelet effects (198) making it less likely that any

potential changes would be platelet-mediated. Salicylates are ubiquitous in food products (199) and the antioxidant properties exhibited by various food groups (Tea, Grapefruit, Wine, etc) may be potential confounding factors. Evidence from studies such as JUPITER (40) and other landmark studies on statins and angiotensin receptor blockers have demonstrated that these classes of drugs have an anti-inflammatory and vascular protective effect (See table-3). This would make it difficult to tease out the influence of aspirin on the pathophysiological factors under study.

Photoplethysmography has been validated for the indirect measurement of endothelial function. It relies on fairly large changes in endothelial reactivity to demonstrate changes in endothelial function in type 2 diabetes compared to healthy volunteers. (177) Thus any small changes in endothelial function with various aspirin doses in this study would have been difficult to detect and interpret. Moreover the 2 week duration may have been insufficient to demonstrate a more significant/graded difference.

While we calculated that the study was designed to detect a 20% change in parameters at the 90% level of confidence, the best estimate of the size of change that can be reasonably excluded from the negative results can be obtained by calculating 95% confidence intervals (CI) for the change in outcome measure after aspirin relative to that after placebo. Such measurements would be useful for future studies in this area. Specific analyses to look for carryover effects between interventions are available but have not been applied in this instance. The study was set-up to give the treatments in a random order to counter the possibility any influence a particular order may have on the outcomes studied. The duration between interventions was also pre-specified to

eliminate any interference between treatments. Therefore specific analyses to look for carryover effects have not been performed.

Section 4 ***Other antiplatelet agents and risk of GI bleeding***

Clopidogrel and Ticlopidine belong to a class of anti-platelets called Thienopyridines which are prodrugs that act on Adenosine Diphosphate (ADP) mediated platelet aggregation via the glycoprotein IIb/IIIa pathway.(200; 201) Clopidogrel is converted via the cytochrome P450 pathway (CYP2C19 being a key isoenzyme) to an active metabolite which inhibits ADP-stimulated activation of platelets via the platelet P_2Y_{12} receptor. (201) Clopidogrel seems to confer better cardiovascular protection compared to aspirin as reported by CAPRIE (137) with an 8.7% relative risk reduction in ischaemic events on an annual basis for patients on clopidogrel versus aspirin. This is for all patients with cardiovascular disease rather than just those with diabetes. A combination of aspirin and clopidogrel has been shown to be better than aspirin or clopidogrel alone in reducing cardiovascular events as secondary prevention (202; 203) and dual anti-platelet therapy has become the standard of care in the secondary care setting especially with regards to coronary heart disease. The use of clopidogrel and aspirin combination when compared to clopidogrel alone has a small but non-significant effect on outcomes in ischaemic stroke and particularly in high risk patients.(204)

In the context of primary prevention however, the CHARISMA study (205) did not find any additional benefit of combining aspirin with clopidogrel compared to aspirin alone. Furthermore it is clear that using combination therapy while potentiating antiplatelet activity confers additional risk of GI bleeding.(204) There is variable evidence as to whether clopidogrel is safer than aspirin in respect of GI complications.(137; 206; 184) A retrospective study cohort has suggested similar results with lower risk of GI bleed with aspirin in combination with a PPI compared to clopidogrel in combination with a PPI.(182) Another more recent cohort study which looked at Clopidogrel alone in combination with Pantoprazole (PPI) or Omeprazole has shown clear benefit of concomitant PPI use in reducing the risk of GI bleeding (207). However, several studies (207-9)-) have raised concerns that the combination of clopidogrel with a PPI, reduces the antiplatelet activity of clopidogrel and leads to an increase in adverse cardiovascular outcomes. For example in the study by Ray et al. (207) for the cohort on combination therapy (clopidogrel and PPI) the hazard ratio (HR) for CV risk was 0.99 (CI-0.82 to 1.19) for the whole cohort and 1.01 (CI-0.76 to 1.34) for the sub-group who had coronary revascularisation and stenting which raises the clinical concern of an increase in cardiovascular risk.

These concerns have led to seeking alternative strategies to this combination including use of H-2 (histamine type 2) receptor blockers such as ranitidine. The basis for such an interaction remains unclear. PPI inhibition of the CYP2C19 cytochrome enzyme, a vital component of the cytochrome P450 pathway needed to convert clopidogrel (a prodrug) to its active metabolite, has been cited as a mechanism.(208) The studies looking into this thus far are plagued by problems with design, confounders which have not been fully accounted for, and potential alternative explanations for the varying efficacy of clopidogrel such as differences in P450 genetic polymorphisms.(210) Despite this potential interaction the effectiveness of clopidogrel, there is still a case for adding in PPI rather than not using clopidogrel and certainly the risk-benefit ratio for PPI use is strongly in favour of such a combination where dual anti-platelet therapy is used or anti-platelet agents are used in combination with anti-coagulants such as warfarin or unfractionated heparin, and in individuals with high risk of GI bleeding.(211)

Section 5 – Directions for future research

Further studies with aspirin using more sensitive markers of oxidative stress induced damage such as isoprostanes and endothelial function, in a bigger population may help elaborate further on these links. Any risk-benefit analysis/study of aspirin at different doses needs to take into consideration the gastro-intestinal and potential renal side effects (especially with widespread use of RAS blockade in diabetes). Larger secondary and primary prevention studies comparing the clinical outcomes with different dosing of aspirin may be able to definitively address optimisation of aspirin dosing. At present there are further studies underway to try and answer

this question such as the JPP study in Japan (212) The NF-κB pathway is vital to several cellular functions and further research is needed to study the influence of aspirin on this pathway.

Recent research has focused on a multipronged approach to risk reduction in diabetes (especially type 2 which is by far the most prevalent type) with the use of a polypill (consisting of aspirin 81mg, enalapril 2.5mg, atorvastatin 20mg, and hydrochlorothiazide 12.5mg) (213) demonstrating definite benefits in blood pressure reduction, improved lipid profile and CV mortality. Dropout rates were higher in the treatment group than the placebo group suggesting that compliance maybe a factor in the modest results demonstrated. Other studies are underway looking at the health economics of such polypill use. Slightly different combinations are proposed with aspirin, statin, and an ACE-inhibitor being a fixed combination alternating with a beta blocker and/or a thiazide, for those with or without previous myocardial infarction respectively (214) Aspirin remains cost-effective and useful in type 2 diabetes (215) and a polypill might be the way forward in terms of global risk reduction in the context of a rapid rise in type 2 diabetes and obesity both of which are harbingers of cardiovascular disease.

The development of NO-aspirins such as NCX-4016, an NO-releasing aspirin, which would potentially be more active in the vasculature (216) and cause fewer G.I. side effects, (96) although appealing, have not progressed into the clinical realm and remain speculative at best.

Section 6 – Conclusions

In summary, the 2 parts of our study was aimed at understanding the role of aspirin in day to day practice in the context of primary and secondary prevention of cardiovascular disease and the effects of different doses in the pathophysiology of type 2 diabetes and specifically in relation to cardiovascular risk factors that form the hallmark of this disease. The observational element of the study, with the caveats of sample size and prescriber status, has highlighted the heterogenous nature of aspirin prescription in a UK healthcare setting. Decades of study have demonstrated the cardiovascular benefits of aspirin with a number of studies ascribing non-plaletet effects to aspirin while underlining its anti-platelet properties. This study into pathophysiology conducted in a population with type 2 diabetes and high cardiovascular risk has not demonstrated any significant differences which would support either facet of aspirin activity and no negative effects of aspirin action were noted on the markers studied. Much bigger studies (focusing on clinical endpoints) have failed to conclusively answer the questions on aspirin use in primary prevention. This study was designed to look for pathophysiological clues which could help inform the bigger picture and point to areas for further large scale research. However, it did not demonstrate a dose-response relationship of aspirin with respect to the factors studied. Good glycaemic control, good blood pressure, and high degree of statin use may have contributed to this seeming lack of effect.

Guidelines for aspirin use especially in the acute situation have changed in recent years to reflect the acute pro-inflammatory state found in situations such as acute MI, thrombotic stroke, and post revascularisation in conditions such as peripheral vascular disease and following stent insertion in coronary artery disease. The observational element of this study confirmed that the controversy in current aspirin guidance was reflected in a heterogeneous prescribing of aspirin in patients with diabetes. The majority of the responses to our survey were from 2 areas in the south of England.

A bigger survey covering the whole of the UK would help to ascertain if this response is representative of the whole of the UK. Further clarification and guidance on the optimum dose of aspirin in diabetes is required and there is need for consistent advice especially in primary prevention. This is important with the advent of newer anti-platelet agents and the continued concerns regarding gastro-intestinal bleeding with anti-platelet therapy. Even newer agents such as clopidogrel and prasugrel are potentially on an equal footing with aspirin with regards to risk of gastrointestinal bleeding and non–GI haemorrhagic events (e.g. haemorrhagic stroke), an important consideration in deciding on anti-platelet therapy particularly in those with diabetes. While combination therapy with newer antiplatelet agents such as clopidogrel confers extra benefit in certain settings, such therapy also increases the risk of GI bleeding.

Recommendations for aspirin use in primary prevention previously included "everyone above 40 years with diabetes" but now are more focused - "those with diabetes age>50 yrs. if male and age>60 yrs. if female with one additional risk factor" (smoking, family history of heart disease, dyslipidaemia, and microalbuminuria or overt proteinuria).(71). The risk of extracranial (mainly upper gastro-intestinal bleeding) should be taken into consideration along with the above factors when recommending aspirin. The lack of effect of aspirin on key pathophysiological factors underlying type 2 diabetes at different doses used in this study, supports the current position of sticking to low dose (≤150mg) aspirin long term and using higher doses (up to 600mg/day), in the acute setting. Barring contraindications and patient wishes, aspirin use in primary prevention should follow the ADA 2010 recommendations consistently.

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APPENDIX A

Aspirin Use in Diabetes- Questionnaire

Place of work: GP partnership/Sole GP/Community Clinic/Secondary Care

Your Role in the Team:

GP/GPSI/GP Associate/DSN-community/DSN-Hospital/Hospital Consultant-Diabetes/Hospital Consultant-Other/Specialist Registrar/Associate Specialist/GP Registrar

For secondary care - Specialty :_____

Your views on Aspirin use in Diabetes: (Please state whether you strongly agree or disagree with the following)

A. In those with Diabetes and SECONDARY Prevention (i.e. history of CVA/TIA, CVD, and PVD) Aspirin should be given to

- **All subjects unless there is a contraindication-**

[1] Strongly agree **[2] Agree** **[3] Neither agree/disagree** **[4] Disagree** **[5] Strongly disagree**

- **All those who have had a cardiac event**

[1] [2] [3] [4] [5]

- **All who have had a stroke or TIA**

[1] [2] [3] [4] [5]

- **All patients who have peripheral vascular disease**

[1] [2] [3] [4] [5]

B. In those with Diabetes and PRIMARY prevention (i.e. without CVD, CVA/TIA, PVD) you would recommend/prescribe aspirin to

- **Everyone in this category**

[1] Strongly agree **[2] Agree** **[3] Neither agree/disagree** **[4] Disagree** **[5] Strongly disagree**

- **Those who are smokers or have a smoking history**

[1] [2] [3] [4] [5]

- **Those who have hypertension**

  [1] [2] [3] [4] [5]
- **Those with hyperlipidaemia**

  [1] [2] [3] [4] [5]
- **Those who have a strong <u>family history</u> of CVD (1st degree relative with CVD < 55 yrs age)**

  [1] [2] [3] [4] [5]
- **Those with strong family history of cardiovascular disease**

  [1] [2] [3] [4] [5]
- **Those who are at > 20% risk of a vascular event over 10 years**

  [1] [2] [3] [4] [5]
- **Those with microalbuminuria**

  [1] [2] [3] [4] [5]

C. <u>Aspirin dose/preparation: Please indicate your views on the following in diabetes , assuming no contraindications exist (please circle your preferred option)</u>

In Diabetic patients:

- **I only recommend one dose of aspirin**

[1] [2] [3] [4] [5]

Strongly agree Agree Neither agree/disagree Disagree Strongly disagree

- **In a patient immediately post-MI the aspirin dose you would use-**

No Aspirin <75mg/d 75mg/d 150mg/d 300mg/d

- **In a patient immediately post CVA**

No Aspirin <75mg/d 75mg/d 150mg/d 300mg/d

- **In a patient who presents immediately post TIA**

No Aspirin <75mg/d 75mg/d 150mg/d 300mg/d

- **In a patient who presents with recurrent TIA**

No Aspirin <75mg/d 75mg/d 150mg/d 300mg/d

- **In a patient who has previous history of -- CVA/TIA/MI/Angina/PVD**

No Aspirin <75mg/d 75mg/d 150mg/d 300mg/d

- **In a patient as primary prevention**

No Aspirin <75mg/d 75mg/d 150mg/d 300mg/d

- **Is there any difference in your use/dose of aspirin between those with type 1 or type 2 diabetes? Yes/No**

If yes, in what way……

- **Do you routinely prescribe enteric coated aspirin?**
  Never/ occasionally/some of the time/ a lot of the time/always
- **Have you had queries from your diabetes patients about the use of aspirin?**
  No queries/ A few/ Some/ Many/ Lots of queries

- **I would routinely recommend PPI cover when giving aspirin?**

[1]	[2]	[3]	[4]	[5]
Strongly agree	Agree	Neither agree/disagree	Disagree	Strongly disagree

- **In a patient with diabetes and history of peptic ulcer disease needing <u>primary</u> prevention you would-**
  - **use aspirin with PPI cover**
  - **use an alternate anti-platelet agent**
  - **use enteric coated aspirin**
  - **not use any anti-platelet agents**

- **In a patient with diabetes and history of peptic ulcer disease needing <u>secondary</u> prevention you would**
  - **use aspirin with PPI cover**
  - **use an alternate anti-platelet agent**
  - **use enteric coated aspirin**
  - **not use any anti-platelet agents**

- **I would take aspirin if I had diabetes?**

Definitely Yes /Depends/ Not Sure/ Definitely No

D. Any Additional Comments: (Free text)

APPENDIX-B

Additional metabolic Influences	Numbers (%) & sub-categories
Anti-hypertensive therapy	Yes=13 (76.5), No=4 (23.5)
Use of anti-hypertensive agents targeting the Renin-Angiotensin system	ACE-i = 11/13 ARB = 2/13 Total = 13 Total anti-hypertensive medications (all groups) = 21
Lipid lowering therapy	Number on therapy=16/17 (94) Statins=15/17 Fibrates=2/17
Smoking status	Yes=4/17 (23), No = 13/17 (77)

Table: Additional factors that may have influenced metabolic parameters
(ACE-i = Angiotensin Converting Enzyme inhibitor, ARB = Angiotensin Receptor Blocker)

Posters, Presentations and Publications:

Poster Presentations	Venue/Journal
Raghavan RP, Laight DW, Page G, Cummings MH. Effect of aspirin titration on markers of systemic and vascular inflammation in type 2 diabetes	DUK 2012, Glasgow Diabetic Medicine, 29 (Supp. 1), P70, March 2012
Raghavan RP, Laight DW, Cummings MH. Aspirin use in diabetes. Survey of a cross-section of healthcare professionals.	Research Away Day, 6th March 2012, Portsmouth Hospitals NHS Trust
Raghavan RP, Laight DW, Cummings MH. Survey of aspirin use amongst healthcare professionals.	ABCD Spring meeting, 6th May 2011, Birmingham
Raghavan RP, Laight DW, Shaw KM, Cummings MH. Does aspirin have a dose dependent effect upon endothelial function in Type 2 diabetes? (short listed- Clinical Science award)	DUK 2008, Glasgow Diabetic Medicine, 25(suppl l), P27, March 2008
Cummings MH, Kar P, **Raghavan RP**, Allard SA, Laight DW, Shaw KM. Correlation between Markers of endothelial function, oxidative stress, Insulin resistance, and glycaemic control in a type-2 diabetes population.	DUK 2008, Glasgow Diabetic Medicine, 25 (suppl 1), P29, March 2008
Raghavan RP, Laight DW, Shaw KM, Cummings MH. Effects of aspirin on oxidative stress in type2 diabetes.	DUK 2007, Glasgow Diabetic Medicine, 24 (s1) , P65, March 2007

Journal Articles:

- **Raghavan RP,** Laight DW, Cummings MH. Effect of aspirin titration on markers of oxidative stress, insulin resistance, dysglycaemia, endothelial function, and vascular inflammation in type 2 diabetes-Manuscript in preparation.
- **Raghavan RP**,.Laight DW, Cummings MH. Survey of Attitudes to Aspirin Use in Diabetes- Practical Diabetes International July 2012
- **Raghavan RP**, Laight DW, Shaw KM, Cummings MH, Aspirin and Diabetes (review article); British Journal of Diabetes & Vascular Disease, 6: 74-82, April 2006